Non-Canonical Wnt Signaling Maintains Hematopoietic Stem Cell Through Flamingo and Frizzled8 Interaction in the Niche

Thesis

UNRESTRICTED

Non-canonical Wnt Signaling Maintains Hematopoietic Stem Cell through Flamingo and Frizzled8 Interaction in the Niche

Ryohichi Sugimura, MD

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Stowers Institute for Medical Research,
an Affiliated Research Centre
of the Open University

5 June 2012

Date of Submission: 24 April 2012
Date of Award: 10 October 2012.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ACKNOWLEDGMENTS

I would like to thank Leanne Wiedemann and Shelly Hornbuckle throughout my program here. I would like to express appreciation to committee members Jerry Workman, Ting Xie, Ron Yu and Jeff Haug. I would like to thank Linheng Li, Xi He, and all the Li lab members who have helped me develop as a scientist. Xi He taught me histology work, immunostaining, and flow cytometry. Aparna Venkatraman helped me dissect niche components. John Perry taught me HSC culture. Linheng Li, as my mentor, guided me on this project, and I have always enjoyed discussion with him.

I thank the following for help with experiments and sharing reagents for my thesis study. Ariel Paulson and Hua Li for RNA-sequencing analysis, and Dr. H. Nakauchi for training in sorted HSC immunostaining. I appreciate Andrew Box, Craig Semerad and Jun Park for flow cytometry assistance, as well as Will Marshal, Dave Dai and Richard Alexander for imaging assistance, and William McDowell and Anoja Perera for help with molecular biology experiments. I appreciate Tari Parmely and her tissue culture team, and Terry Johnson and the histology core team. I appreciate Dr. G. Enikolopov, Dr. D. Rowe, Dr. V. Bautch, Dr. E. Fuchs, Dr. F. Costantini and Dr. T. Xie for providing respectively these mouse lines: Nestin-GFP, Col2.3-GFP, CD31-ESCs, TOP-GAL, Axin2-d2EGFP, and pSicoR-EF1α. I am grateful to John Perry and other Li lab members for scientific discussion and manuscript critical reading; to Mark Hembree, Kim Westpfahl, Debra Dukes, and Brandy Lewis for technical support; and to Karen Tannen for thesis editing and proofreading.

ABSTRACT

Wnt signaling is involved in self-renewal and maintenance of hematopoietic stem cells (HSCs); however, the particular role of non-canonical Wnt signaling in regulating HSCs *in vivo* is largely unknown. Here I show Flamingo and Frizzled8, members of non-canonical Wnt signaling, both express in and functionally maintain quiescent long-term HSCs. Flamingo regulates Frizzled8 distribution at the interface between HSCs and N-cadherin^{+} osteoblasts (N-cad^{+}OBs that enrich osteoprogenitors) in the niche. I further show that N-cad^{+}OBs predominantly express non-canonical Wnt ligands and inhibitors of canonical Wnt signaling under homeostasis. This non-canonical Wnt signaling is attenuated prior to activation of HSCs. In the activated HSCs, however, canonical Wnt signaling is enhanced. Mechanistically, non-canonical Wnt signaling mediated by Frizzled8 suppresses the Ca^{2+}-NFAT- IFNγ pathway and antagonizes canonical Wnt signaling in HSCs. My findings demonstrate that non-canonical Wnt signaling maintains quiescent long-term HSCs through Flamingo and Frizzled8 interaction in the niche.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

5FU	5-fluorouracil
7AAD	7-aminoactinomycin D
AKT	v-akt murine thymoma viral oncogene homolog
APC	Adenomatous polyposis coli
Bcl	B-cell lymphoma
BCR-ABL	Break point cluster region-Abelson
BM	Bone marrow
BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine
CA-NFAT	constitutive active form of NFAT
CaMK	Calmodulin-dependent kinase
CAR cell	Cxcl12-abundant reticular cell
CBR	Compact bone region
Ccnd	Cyclin D
CD	Cluster of differentiation
Cdc42	Cell division cycle 42
Cdk	Cyclin-dependent kinase
Cdkn	Cyclin-dependent kinase inhibitor
Celsr	Cadherin EGF LAG 7-path G type receptor
CFDA	Carboxyfluorescein Diacetate
CK1α	Casein Kinase 1 alpha
CLL	Chronic lymphoid leukemia
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
CMV	Cytomegalovirus
Col	Collagen
COX	Cyclooxigenase

CXCR	C-X-C chemokine receptor
DAAM1	Dishevelled associated activated morphogenesis 1
DAPI	4'6-diamidino-2-phenylindole
Dkk	Dickkopf
Dox	Doxycycline
DSCR1	Down syndrome critical region gene 1
Dvl	Disheveled
DYRK1A	Dual specificity tyrosine-phosphorylation-regulated kinase 1A
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FITC	Fluoroscein Isothiocyanate
FLK2	Fetal liver kinase 2
Fmi	Flamingo
FOX	Forkhead box
FPKM	Fragments per kilobase of exon per million
Fz	Frizzled
GAL	Galactosidase
GFP	Green fluorescent protein
GPCR	G-protein-coupled receptor
GRP78	Glucose-regulated protein of 78 kD
GSK	Glycogen Synthase Kinase
GTP	Guanosine Triphosphate
H2B	Histone H2B
HOX	Homeobox
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem/progenitor cell
IFN	Interferon
IL	Interluekin

IRES	Internal ribosome entry site
ISC	Intestinal stem cell
JNK	c-Jun N-terminal Kinase
FPKM	Fragments per Kilobase of exon per Million fragments
LEF	Lymphoid enhancer factor
LRC	Label-retaining cell
LRP	Lipoprotein receptor-related protein
LSK	$Lineage^{-}Sca1^{+}cKit^{+}$
LT-HSC	Long-term hematopoietic stem cell
MAPK	Mitogen-activated protein kinase
MMTV	Mouse mammary tumor virus
MPP	Multipotent progenitor
MSC	Mesenchymal stem cell
Mx1	Myxovirus resistance 1
Mxd1	MAX dimeralization protein 1
N-cad	N-cadherin
$N\text{-}cad^{+}OBs$	$N\text{-}cadherin^{+}$osteoblasts
NFAT	Nuclear factor of activated T-cell
NFκB	Nuclear factor kappa B
NLK	Nemo-like kinase
OB	Osteoblast
PAR1	Prader-Willi/Angelman region-1
PBS	Phosphate buffered saline
PBS/2%FBS	Phosphate buffered saline with 2% fetal bovine serum
PCP	Planar Cell Polarity
PCR	Polymerase chain reaction
PDE	Phospho di esterase
PE	Phycoerythrin
PI3K	Phosphoinositide 3-kinase
PKC	Protein Kinase C

PLC	Phospholipase C
PPAR	Peroxisome proliferator-activator receptor
PTEN	Phosphotase and tensin homolog
Ptprc	Protein tyrosine phosphatase, receptor type, C
qRT	Quantitative reverse transcription
Rac2	Ras-related C3 botulinum toxin substrate 2
Rbl2	Retinoblastoma-like 2
ROR	Receptor-tyrosine-kinase-like orphan receptor
SCA1	Stem cell antigen 1
SCF	Stem cell factor
SCL	Stem Cell Leukemia
SFRP	Soluble Frizzled-related protein
shRNA	Short-hairpin RNA
ST-HSC	Short-term hematopoietic stem cell
TAK1	Transforming growth factor-β-activated kinase
Tal1	T-cell acute lymphocytic leukemia 1
TBR	Trabecular bone region
TCF	T-cell factor
TK	Thymidine kinase
TPO	Thrombopoietin
TRE	Tetracycline-responsive element
Treg cell	Regulatory T cell
tTA	Tetracycline transactivator
Txnip	Thioredoxin-interacting protein
Vangl2	Vang-like 2
VEGFR	Vascular endothelial growth factor receptor
WIF	Wnt inhibitory factor

Chapter 1. Introduction

In this chapter, I will present the hematopoietic stem cell (HSC) and its associated cellular components, termed "niches", and the signals emanating from the niches, with a particular emphasis on Wnt signaling.

1.1 Stem Cells

Stem cells are a population of cells with the ability to self-renew and the pluripotential or multipotential to give rise to all the different cells in a body (embryo) or in a given tissue (adults). The regulation of stem cells is crucial to support embryo development, establishment of organs, and homeostasis of tissues in the body. The disruption of stem cell regulation often leads to diseases including tissue defects or cancer (Perry and Li, 2007; Reya and Clevers, 2005; Yilmaz et al., 2006a; Zhang et al., 2006). Accumulated evidence reveals that extrinsic signaling is important for the maintenance, proliferation, and lineage fate determination of stem cells. Among the various types of extrinsic signaling, Wnt signaling is one of the best characterized pathways to regulate stem cells (Reya & Clevers, 2005).

1.2 Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are multipotent stem cells that give rise to all the blood cell types (Weissman et al., 2001). HSCs are comprised of long-term (LT)-HSCs, short-term (ST)-HSCs, and differentiate to multipotent progenitors (MPPs). LT-HSCs are enriched with quiescent HSCs and function over 16-44 weeks when transplanted to mice. In contrast, ST-HSCs are enriched with actively cycling HSCs and can sustain hematopoiesis for only 4-6 weeks, ending up with loss of myeloid cells first, followed by loss of lymphoid cells (Benveniste et al., 2010). MPPs are

transient-amplifying cells and differentiate to either myeloid or lymphoid lineages (Larsson and Karlsson, 2005) (Figure 1-1). HSC is the best characterized stem cell, and studies of HSCs have revealed new concepts and functions regarding their maintenance and regulation. Furthermore, the insights from HSC study can lead to improvements in clinical practice, such as bone marrow (BM) transplantation and targeting cancer stem cells.

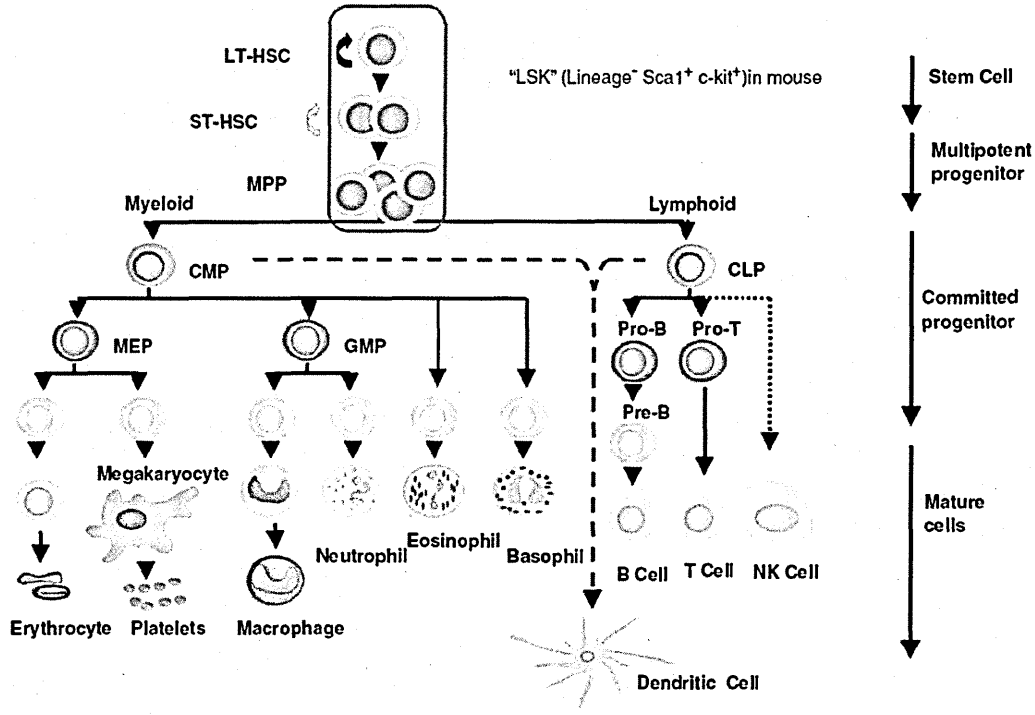

Figure 1-1 The process of hematopoiesis in adults.

HSCs give rise to multipotent progenitors (MPPs) which further differentiate to common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). (Adapted from Larsson & Karlsson, 2005.Oncogene)

1.3 HSCs in Development, Adult and Aging

Recently it was shown that HSCs are *de novo* generated from endothelial cells located in Aorta-gonad-mesonephros (AGM) at E10.5 (in mouse) (Boisset et al., 2010). After E12.5, HSCs migrated to fetal liver where HSCs underwent expansion

and reached a peak during E15.5-16.5. Around E17.5, fetal HSCs seeded to BM (Mikkola and Orkin, 2006). After birth, BM became the main hematopoietic organ through the rest of adult life. Under stress, however, HSCs can migrate to spleen or potentially to liver and undergo extramedullary hematopoiesis. A portion of HSCs in adult mice are in the quiescent state for long-term maintenance, and a portion of HSCs are in cycling to support blood production. A regulation of the balance between the quiescent and proliferating states of HSCs is critical. Loss of the balance can result in either HSC exhaustion or HSC aging. For example, in adult mice (>2 years), HSCs have a feature of an increased quiescence (cannot be activated) with a bias towards myeloid and reduced lymphoid lineages.

The underlying mechanism of HSC aging is actively investigated in the stem cell field. A recent report showed that an increase of Cdc42 activity is responsible for aging of HSCs. When adult HSCs were treated with Cdc42 inhibitor, they became rejuvenated (Florian et al., 2012). Additionally, Eaves and colleagues reported 2 types of HSCs: α-HSC (lymphoid deficient) and β-HSC (balanced lineage). In fetal liver, β-HSC is predominant. In contrast, α-HSC becomes predominant in BM with aging (Benz et al., 2012). However, the mechanisms that regulate the transition of HSC state and correlation with potential changes in the associated niches are not clear.

1.4 The Stem Cell Niche

1.4.1 Stem cell niches in different tissues

In 1978, Ray Schofield proposed a concept of stem cell niche. According to this concept, stem cells are not randomly distributed but rather located in a specified microenvironment where specific extrinsic signals play a role in maintaining stem

cells at an undifferentiated state (Schofield, 1978). Another 20 years passed before the stem cell niche was identified in 1998 when it was first found in Drosophila ovary where germ line stem cells reside (Xie and Spradling, 1998). Our lab together with another lab independently identified mammalian HSC niche in BM using genetic models. (Calvi et al., 2003; Zhang et al., 2003a). For long-term maintenance of adult stem cells, a subset of stem cells needs to be kept in the long-term quiescent state in a specialized niche (Arai et al., 2004; Cotsarelis et al., 1990; Haug et al., 2008; Li and Clevers, 2010; Wilson et al., 2008; Zhang et al., 2003a). Quiescent long-term HSCs are mainly located in the endosteum of the trabecular bone region (TBR), where HSCs are found directly attached to N-cad^{+}OBs known to enrich osteoprogenitors (Wilson et al., 2008; Xie et al., 2009; Zhang et al., 2003a). The key signals emanating from this niche to maintain HSC quiescence, however, remain largely unknown (Li and Clevers, 2010).

The endosteal bone surface is covered by bone-lining cells which include pre-osteoblasts and a specific type of macrophages (osteomacs) (Hauge et al., 2001; Raggatt and Partridge, 2010). Pre-osteoblasts are derived from mesenchymal stem cells (MSCs) and can differentiate into mature osteoblasts to form bone.

1.4.2 Other components of hematopoietic stem cell niches

Recent studies of HSCs have revealed that the endosteal region is a niche to maintain quiescent HSCs and that N-cadherin^{+} bone-lining pre-osteoblasts are one of the major components of this niche (Xie et al., 2009; Zhang et al., 2003a). Many adhesion molecules and signaling molecules have been reported to affect the maintenance of quiescent HSCs in the endosteal niche (Wilson and Trumpp, 2006) . Once HSCs are activated by extrinsic signaling, they will mobilize from the

endosteal niche to central marrow (Levesque et al., 2010). Canonical Wnt signaling is one of the factors known to activate HSCs as well as to prompt bone-lining pre-osteoblasts to differentiate into mature osteoblasts (Malhotra and Kincade, 2009).

MSCs are able to give rise to osteoblasts, chondrocytes, and adipocytes in BM (Pittenger et al., 1999). Transplantation of MSCs showed improved engraftment of hematopoietic cells, implying that differentiated mesenchymal cells in BM support hematopoiesis (Drouet et al., 2005).

Recent reports have identified other niche components in the perivascular zone, such as Nestin$^+$ MSCs (Mendez-Ferrer et al., 2010), Leptin receptor$^+$ perivascular cells (Ding et al., 2012), nonmyelinating Schwann cells (Yamazaki et al., 2011), and CXCR12 abundant reticular (CAR) cells (Sugiyama et al., 2006) (Figure 1-2). In contrast to the endosteal zone where HSCs are kept in quiescence, the perivascular cells maintain HSCs in an active state (Perry and Li, 2012). However, the molecular mechanisms that regulate the transition of HSCs between the endosteal zone and the perivascular zone remain unclear.

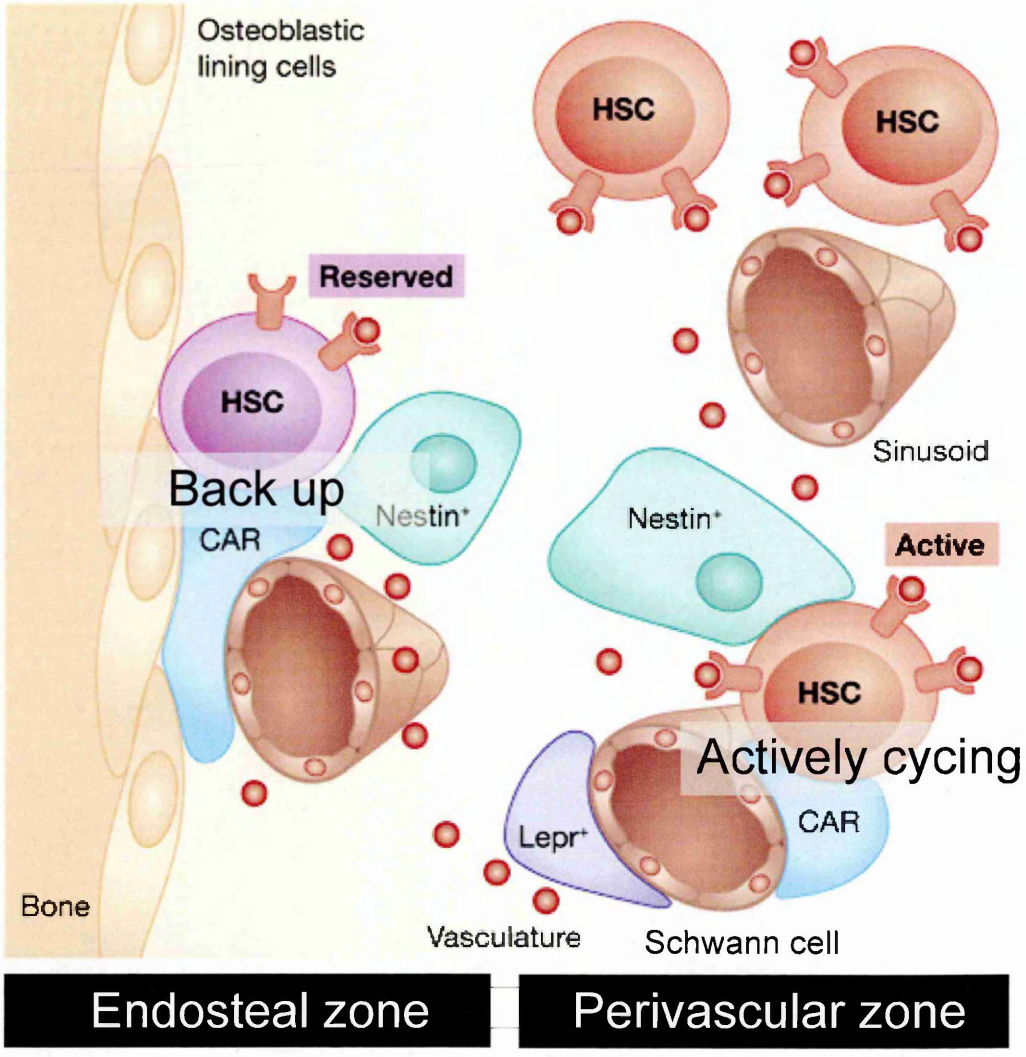

Figure 1-2 HSC niche in zones.

Reserved HSCs are adjacent with N-cadherin$^+$ osteobpasts as well as CAR cells and Nestin$^+$ cells in the endosteal zone. Active HSCs are located in the perivascular zone where sinusoidal endothelial cells, Leprin receoptor (Lepr)$^+$ cells, CAR cells and Nestin$^+$ cells exist. (Modified from Perry and Li, 2012. EMBO Journal)

1.4.3 Bone remodeling and HSC niche in the endosteal zone

Bone is a dynamic tissue that undergoes continual remodeling by osteoblasts and osteoclasts. Osteoclasts are terminally differentiated monocytes which remove mineralized bone matrix. Osteoblasts are specialized bone-forming cells derived from MSCs (Raggatt and Partridge, 2010).

One of the questions to address in studying the osteoblast niche is what happens to this niche during bone remodeling. Our lab previously has shown that N-cadherin is expressed in a specific subset of pre-osteoblasts (N-cad$^+$OBs) (Zhang et al., 2003a), to which BrdU label-retaining quiescent HSCs are attached. In my preliminary data, N-cad$^+$OBs are quiescent. Their position in TBR and bone-lining cell appearance suggests that N-cad$^+$OBs may be located on the "inactive bone surface" and do not engage in bone remodeling, but can be activated upon large loss of mature osteoblasts (Miller and Jee, 1987). It is interesting to conduct a lineage-tracing starting from N-cad$^+$OBs to determine whether they contribute to bone remodeling.

1.4.4 Bone remodeling and Wnt signaling

Canonical Wnt signaling is a key pathway in bone formation. The activation of β-catenin through co-receptor LRP5/6 induces osteoblast formation and high bone mass (Boyden et al., 2002). Non-canonical Wnt signaling regulates commitment of MSCs to the osteoblasts (Baksh and Tuan, 2007). In the following sections, I will describe molecular components and function of canonical and non-canonical Wnt signaling.

1.5 Canonical Wnt Signaling

Wnt signaling plays a central role in many processes during development and adult stages of life, and its abnormality is involved in a variety of diseases (Logan and Nusse, 2004). Wnt signaling can be broadly categorized as canonical and non-canonical (Nelson and Nusse, 2004; Veeman et al., 2003).

In 1982, Nusse and Varmus identified the mouse proto-oncogene *Integration 1* (*Int1)* (now designated as *Wnt1*) in the breast tumors of mice infected with mouse

mammary tumor virus (MMTV). Int was identified as a vertebrate gene near several integration sites of MMTV (Nusse and Varmus, 1982). The name of Wnt came from Wg (Wingless) and Int in Drosophila. Wg was originally identified as a recessive mutation affecting wing and haltere (appendage behind the wing) development in Drosophila (Sharma and Chopra, 1976), and then Wg was identified as a homolog of Int1. Canonical Wnt signaling controls the stability of β-catenin. In the absence of Wnt ligands, a β-catenin degradation complex (comprising two ser/thr kinases: GSK-3 and CK-1, and two scaffold proteins: Axin and APC) promotes degradation of β-catenin. Wnt receptor, Frizzled, is a family of 7-path transmembrane molecules and G-protein coupled receptors. Wnt ligands bind to Frizzled receptors. The lipoprotein receptor-related protein (LRP) family is well defined for its role in mediating the interaction between canonical-Wnt and Frizzled. When Wnt ligands engage a cognate receptor Frizzled along with a LRP 5/6 co-receptor, the Dishevelled (Dvl) protein is recruited to the plasma membrane. Dvl is a central component to mediate downstream events of both canonical and non-canonical Wnt signaling. Wnt binding to Frizzled protein recruits Dvl to the plasma membrane, which leads to activation of downstream pathways. Different domains within Dvl, including DIX, PDZ and DEP, diverge different downstream pathways (Habas and Dawid, 2005). DIX and PDZ domains function together in canonical Wnt signaling to stabilize β-catenin. DIX domain binds with Axin and results in inhibition of the β-catenin degradation complex in canonical Wnt signaling (Kishida et al., 1999). Phosphorylated β-catenin at C-terminal Serine552 by Akt (He et al., 2007) is translocated to the nucleus where it interacts with members of the T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors to regulate gene expression. The signaling regulates embryo

body axis and proliferation of cells (Liu et al., 1999; Pinto et al., 2003). The transcriptional targets of β-catenin-TCF complex include cMyc, Cyclin D and Axin2. cMyc and Cyclin D positively regulate cell-cycle. Axin2 is a negative regulator of canonical Wnt signaling (Jho et al., 2002) (Figure 1-3).

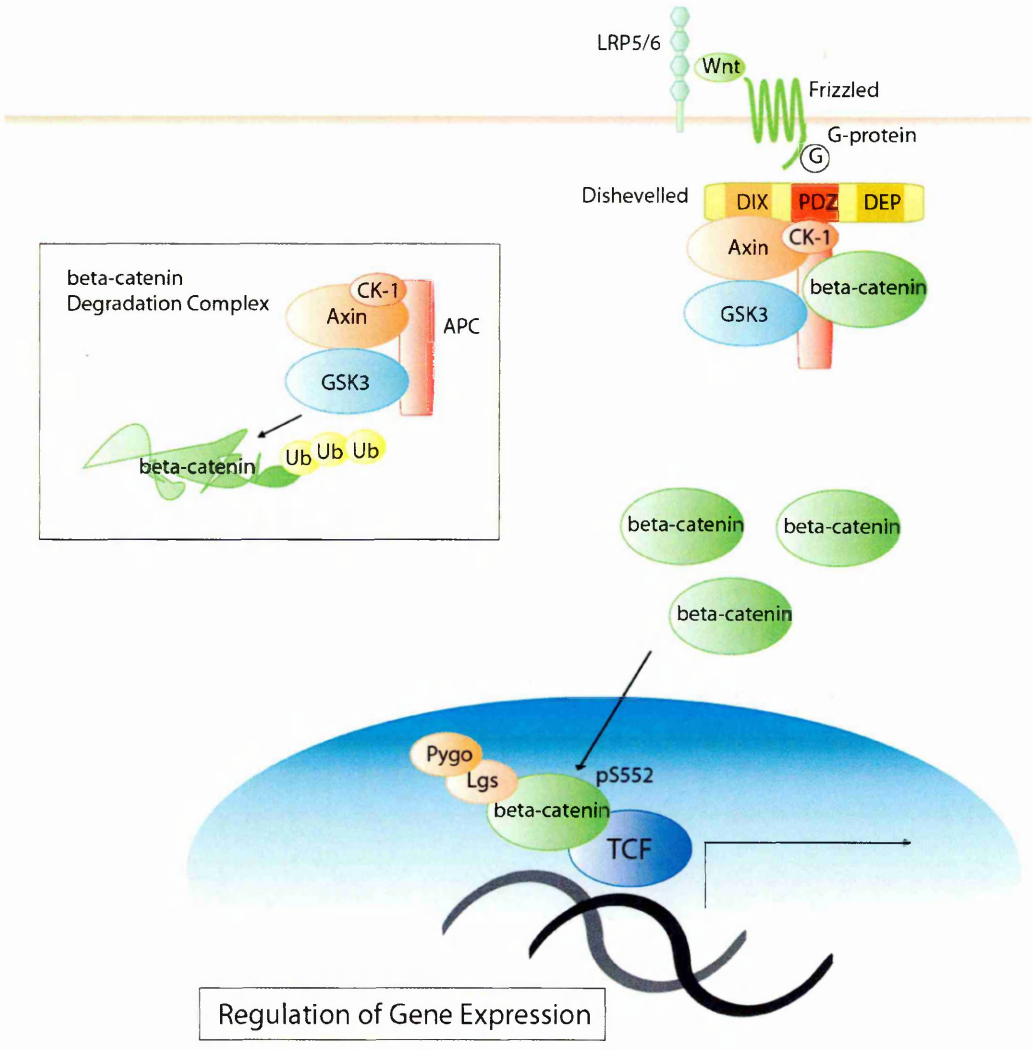

Figure 1-3 Canonical Wnt signaling

In the absence of Wnt ligand, β-catenin is degraded by a complex composed from Axin, APC, CK-1 and GSK3. Once Wnt ligand is bound with Fz and LRP5/6 co-receptor, Dishevelled scaffolds β-catenin degradation complex resulting in accumulation of β-catenin in cytosol and nucleus. β-catenin phosphorylated at S552 (He et al., 2007) and translocated to the nucleus where it formed a complex with TCF, Lgs and Pygo to transcribe target genes. (Modified from Sugimura & Li, Birth Defect Research Part C, 90: 243-256, 2010)

1.5.1 Canonical Wnts

Canonical Wnt ligands include the following: Wnt2 (Sousa et al., 2010), Wnt2b (Goss et al., 2009), Wnt3 (Bhat et al., 2010), Wnt3a (Nygren et al., 2007), Wnt7a (Ohira et al., 2003), Wnt7b (Wang et al., 2005b), Wnt8a (Lindsley et al., 2006), Wnt8b (Lee et al., 2006), Wnt9a (Spater et al., 2006), Wnt9b (Lan et al., 2006), Wnt10a (Gelebart et al., 2008), Wnt10b (Longo et al., 2004) (Table 1-1).

1.5.2 Canonical receptor Frizzleds

The receptors that mediate canonical Wnt signaling include: Fz1 (Bhat et al., 2010), Fz2 (Li et al., 2008), Fz4 (Planutis et al., 2007), Fz9 (Ranheim et al., 2005), Fz10 (Wang et al., 2005b) (Table 1-1)

Table 1-1 Canonical Wnt signaling

Canonical Wnt ligands	
Wnt2	Sousa, 2010
Wnt2b	Goss, 2009
Wnt3	Bhat, 2010
Wnt3a	Nygren, 2007
Wnt7a	Ohira, 2003
Wnt7b	Wang, 2005
Wnt8a	Lindsley, 2006
Wnt8b	Lee, 2006
Wnt9a	Spater, 2006
Wnt9b	Lan, 2006
Wnt10a	Gelebart, 2008
Wnt10b	Longo, 2004
Canonical Frizzleds	
Fz1	Bhat, 2010
Fz2	Li, 2008
Fz4	Planutis, 2007
Fz9	Ranheim, 2005
Fz10	Wang, 2005
Co-receptor	
LRP6	Mao, 2001
Mediator	
Dvl	Kinoussi, 2002
GSK-3β	Hur, 2010

Axin	Hsu, 2001
APC	Nishisho, 1991
CK1α	
Transcription factor	
β-catenin	Grass II, 2005
TCF	Staal, 2000
LEF	DasGupta, 1999

1.5.3 Role of Canonical Wnt signaling *in vivo*

Canonical Wnt signaling is involved in the regulation of many events such as embryonic development, morphogenesis, cell proliferation, and lineage fate determination. Wnt3 is expressed in the primitive streak in the early mouse embryo, and Wnt3 mutants display gastrulation defects (Liu et al., 1999). Wnt1 has redundancy with other Wnts. For example, Wnt1 knockout showed deficiency in neural crest derivatives, reduction in dorsolateral neural precursors in the neural tube (with Wnt3a knockout) (Ikeya et al., 1997), and decline in thymocyte number (with Wnt4 knockout) (Mulroy et al., 2002). Wnt7a defect caused female infertility and delayed maturation of synapses in cerebellum (Ciani et al., 2011). Wnt7b knockout showed placental development defect and respiratory failure (Parr et al., 2001).

1.5.4 Classification of Wnts and Frizzleds

In this study, I have classified Wnts and Frizzleds into canonical and non-canonical according to previous literature with *in vivo* model. Wnt3 and Wnt3a have been well characterized as canonical Wnt. Wnt5a, Wnt5b, Wnt11 and Wnt16 have been well characterized as non-canonical Wnts. Some Wnts and Frizzleds can function for either canonical or non-canonical depending on tissue context. In this case, Wnts and Frizzleds have been classified according to evidence in hematopoietic system or cancer. For example, Frizzled8 has been documented as non-canonical

Wnt receptor in chronic myeloid leukemia (CML) (Gregory et al., 2010). In the following sections, I will describe non-canonical Wnt signaling.

1.6 Non-canonical Wnt Signaling

1.6.1 Non-canonical Wnt signaling components

1.6.1.1 Ligand - Wnts

Through their efforts to identify homologs of *Wnt1*, Moon and colleagues identified *Wnt5a* in Xenopus, which turns out to regulate non-canonical Wnt signaling (Christian et al., 1991). In contrast to canonical Wnt1 injection which led to duplication of the embryonic axis in Xenopus, Wnt5a injection led to developmental defects of the head and tail resulting from a perturbation of cellular movements, without inducing ectopic axis. This implied that Wnt5a signaling differs from canonical Wnt signaling (Moon, 1993). Using zebrafish, Moon and colleagues showed that ectopic expression of Xenopus *Wnt5a* increased intracellular concentration of calcium ion (Slusarski et al., 1997). Furthermore, they proved that stimulation of calcium signaling phenocopied Wnt5a signaling. Following this discovery of non-canonical Wnt signaling, various researchers identified multiple contributing Wnt ligands and Frizzled receptors (Table 1-2).

Table 1-2 Ligands of non-canonical Wnt signaling

Non-canonical Wnt ligands	
Wnt1	You, 2004
Wnt4	Chang, 2007; Heinonen, 2011
Wnt5a	Wallingford, 2001
Wnt5b	Hardy, 2008
Wnt6	Schmidt, 2007
Wnt11	Flaherty, 2008
Wnt16	Clements, 2011

Wnt5a is one of the major ligands responsible for non-canonical signaling. In addition to embryo development, non-canonical Wnt signaling regulates tissue development and homeostasis. For example, it recently became known that non-canonical Wnt signaling is involved in regulation of bone formation (Piters et al., 2008). Osteoblasts derived from MSCs (MSCs) are responsible for bone formation. Because MSCs can differentiate to both osteoblasts and adipocytes, regulation may be required to balance the lineage choices. PPAR-γ and Runx2 transcriptional factors are key regulators that determine respectively the lineage fate of MSCs to adipocytes or osteoblasts (Sugimura and Li, 2010). How PPAR-γ and Runx2 expression are regulated is an important question. Kato and colleagues showed that Wnt5a suppresses transcription of PPAR-γ and activates the expression of Runx2 in ST2 mesenchymal progenitor cell line (Takada et al., 2007). A mouse model with haploinsufficiency of Wnt5a revealed bone loss with enhanced adipogenesis in BM. Non-canonical Wnt signaling has been implicated in regulating the balance between osteogenesis and adipogenesis through regulation of PPAR-γ and Runx2 (Kang et al., 2007). However, it remains unknown whether and how non-canonical Wnt signaling interacts with canonical Wnt signaling. To examine this, Tuan and colleagues asked how MSCs in BM are regulated by canonical and non-canonical Wnt signaling (Baksh and Tuan, 2007). They showed that Wnt5a is required for the maintenance of MSCs under the culture condition mimicking BM niche. In contrast, when MSCs were cultured with Wnt5a directly in a plastic dish, MSCs showed enhanced osteogenesis. Wnt3a, however, suppressed osteogenic differentiation but favored adipogenesis (Boland et al., 2004). Furthermore, canonical Wnt signaling suppressed

osteogenesis, which indicates opposing aspects of canonical and non-canonical Wnt signaling.The evidence has shown a dual role of non-canonical Wnt signaling: to maintain MSCs and to induce osteoblastic differentiation.

Since non-canonical Wnt signaling is required for development of many organs through regulation of cell motility, signal disruption may lead to organ malformation. Indeed, supporting evidence of such has been provided in studies of transgenic or knockout mice (Luo et al., 2007; Moon et al., 2004; van Amerongen and Berns, 2006). Wnt11 is required for kidney development, and its knockout showed defect of ureteric branching and consequent kidney hypoplasia in newborn mice (Merkel et al., 2007). Wnt11 also regulates migration of Xenopus neural crest cells, which are multipotent cells that produce neurons, glial cells, melanocytes, and others. Non-canonical Wnt signaling guides this migration to place those cells in the proper microenvironments (Matthews et al., 2008). Recent work by Travers and colleagues identified that Wnt16 induces HSC *de novo* formation in zebrafish through somatic expression of Notch ligands deltaC and deltaD (Clements et al., 2011).

Another ligand of non-canonical Wnt signaling, Wnt4, promotes female gonadal development by blocking the synthesis of gonadal androgens in female embryos. Interestingly, an XY intersex patient with ambiguous genitalia was found to carry a duplication of chromosome 1p35 where Wnt4 is located. Transgenic mice with a human Wnt4 locus (as an extra copy of Wnt4 locus) showed abnormal testicular vasculature, and their testosterone synthesis was inhibited (Jordan et al., 2003).

1.6.2 Non-canonical Wnts

Noncanonical Wnts include: Wnt1 (You et al., 2004), Wnt4 (Chang et al., 2007; Heinonen et al., 2011), Wnt5a (Wallingford et al., 2001), Wnt5b (Hardy et al., 2008), Wnt6 (Schmidt et al., 2007), Wnt11 (Flaherty et al., 2008), Wnt16 (Clements et al., 2011).

1.6.2.1 Receptors - Frizzleds

To understand the role of non-canonical Wnt signaling in neurogenesis, Nathans and colleagues knocked out both Frizzled3 (Fz3) and Frizzled6 (Fz6), which are receptors of non-canonical Wnt signaling (Wang et al., 2006). Fz3 is required for axonal outgrowth and guidance of the central nervous system (Lyuksyutova et al., 2003; Wang et al., 2002). Fz6 regulates hair patterning in skin (Guo et al., 2004). Since substantial redundancy in Frizzled genes had been previously reported in Drosophila (Bhanot et al., 1999; Chen and Struhl, 1999; Kennerdell and Carthew, 1998), Nathans et al suspected that Fz3 and Fz6 functionally interact with each other. Their double mutant mouse (knockout of Fz3 and Fz6) indeed showed a defect of neural tube closure while neither of the single mutants showed the phenotype. This suggests that both molecules redundantly regulate neural tube development.

Frizzled regulates development as well as adult tissue homeostasis, e.g. cardiac hypertrophy: the thickening of cardiac muscle in response to an increased work load. One report showed that Frizzled2 (Fz2) was upregulated during development of cardiac hypertrophy (Blankesteijn et al., 1996) Also, re-expression of Fz2 in cardiac hypertrophy of genes associated with fetal development has been well documented (van Gijn et al., 2002) (Table 1-3).

1.6.3 Non-canonical Frizzleds

The receptors that mediate non-canonical Wnt signaling include Fz3 (Rasmussen et al., 2001), Fz5 (van Es et al., 2005), Fz6 (Heinonen et al., 2011), Fz7 (De Calisto et al., 2005), Fz8 (Gregory et al., 2010)

Table 1-3 Receptors of non-canonical Wnt signaling

Non-canonical Frizzleds	
Fz3	Rasmussen, 2001
Fz5	van Es, 2005
Fz6	Heinone, 2011
Fz7	De Calisto, 2005
Fz8	Gregory, 2010

1.6.3.1 Co-receptor – Flamingo

Co-receptors for non-canonical Wnt signaling include Flamingo or Cadherin EGF LAG 7-path G type receptor (Celsr) 1,2 ,3 and Vangl2 (see Table 1-4).

1.6.4 Function of Flamingo

Recent studies have revealed other types of receptor molecules involved in non-canonical Wnt signaling. One of them is the Flamingo family. Flamingo is a type of atypical cadherin molecule, which has both cadherin repeat domain and G protein-coupled receptor (GPCR) domain, and hence is named Cadherin EGF LAG 7-path G type receptor (Celsr). Flamingo was initially shown in Drosophila to mediate non-canonical Wnt signaling with Frizzled proteins (Usui et al., 1999). The functional interaction of Flamingo with Frizzled raises the question whether these proteins directly interact with each other. A recent study showed that Flamingo

indeed binds with Frizzled, and the resulting protein complex mediates non-canonical Wnt signaling (Chen et al., 2008). The Flamingo protein family includes several isoforms in mammals: Celsr1, 2, and 3 (Tissir et al., 2002), which have been shown to interact with Frizzled proteins (Devenport and Fuchs, 2008; Tissir et al., 2005) (Table 1-4). Vangl2 is another component of non-canonical Wnt signaling shown to interact with Flamingo and Frizzled proteins (Devenport and Fuchs, 2008; Tissir et al.).

Table 1-4 Co-receptors of non-canonical Wnt signaling

Co-receptors	
Celsr1	Tisser, 2002
Celsr2	Tisser, 2002
Celsr3	Tisser, 2002
Vangl2	Davenport &Fuchs, 2008

1.6.5 Mechanism of Flamingo function

The functional role of G-protein in Flamingo (Fmi) has not been characterized. A recent report showed that the downstream molecular mechanism of Flamingo subtype Celsr3 is to regulate intracellular Ca^{2+} level; however, it was not made clear whether G-protein is involved in this process (Shima et al., 2007) (Figure 1-4).

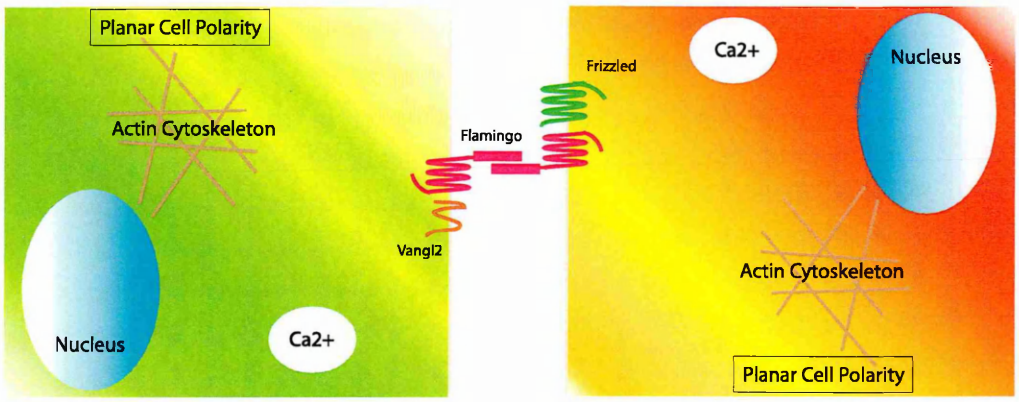

Figure 1-4 Flamingo signaling

Homophilic binding of Fmi between each cell initiates signaling through Fz and Vangl2 to establish polarity of cells. The contribution of calcium is suggested; however, its exact location in downstream pathways is not known. (Modified from Sugimura & Li, Birth Defect Research Part C, 90: 243-256, 2010)

The Flamingo family protein regulates cell polarity through cell-cell contact (Chen et al., 2008; Usui et al., 1999). It affects morphogenesis of neurons and skin keratinocytes (Devenport and Fuchs, 2008; Tissir et al., 2005). Goffinet and colleagues showed Celsr3 is required for neural growth and neural axon guidance in brain development (Tissir et al., 2005). They found Fz3 expressed together with Celsr3 in developing neurons. The single knockout of Celsr3 showed the similar phenotype as Fz3 defect, suggesting that Celsr3 and Fz3 function together to guide the neural axon. The double knockout of both Celsr2 and Celsr3 impaired the membrane distribution of Fz3 (Tissir et al., 2010). This implies that Flamingo (Celsr2 and Celsr3) may regulate the membrane distribution of Fz3, which in turn mediates non-canonical Wnt signaling.

Fz6 regulates hair patterning in skin, a typical function of non-canonical Wnt signaling initially revealed in Drosophila. Recently, Fuchs and colleagues reported that Celsr1 is required to establish polarity of developing hair follicles (Devenport

and Fuchs, 2008). Celsr1, Fz6 and Vangl2 proteins were asymmetrically localized in hair germs. Interestingly, Fz6 required Vangl2 for its asymmetric localization. In addition, Celsr1 recruited Fz6 to cell-cell contacting sites. This suggested that Fz6 shares a common pathway with Celsr1 and Vangl2. Considering the finding that Celsr1 forms calcium-dependent intracellular interactions necessary for recruiting Vangl2 and Fz6, the researchers proposed that Celsr1 homodimer at the adherent site of two cells may initiate the hierarchical events leading to an induction of planer cell polarity (PCP) components through Vangl2 and Fz6. Thus, Flamingo interacts with Frizzled to mediate non-canonical Wnt signaling in a cell adhesion dependent manner.

Mechanistically, Fmi proteins homophilically adhere neighboring cells and determine the polarized or focal distribution of Frizzled protein on the surface of the cell, allowing local interaction with Wnt ligands (Beall et al., 2005; Lee et al., 2003; Tissir et al., 2010). Furthermore, Fmi homophilic junction via cadherin domain mediates contact inhibition as shown in control of dendrite overgrowth (Kimura et al., 2006). Whether Fmi and Fz8 play a role in regulating HSCs has yet to be determined.

1.6.6 Dishevelled-JNK pathway

Non-canonical Wnt signaling antagonizes β-catenin-dependent canonical Wnt signaling by either or both CaMKII-TAK1-NLK pathway and NFAT-mediated transcriptional regulation. CaMKII-TAK1-NLK pathway inhibits β-catenin-TCF dependent transcription through phosphorylation of TCF (Ishitani et al., 2003). NFAT suppresses β-catenin-dependent transcription (Saneyoshi et al., 2002) (Figure

1-5, Table 1-1). The detailed components and function of the downstream pathways will be discussed below.

PDZ and DEP domains of Dvl cooperate in different sub-pathways of non-canonical Wnt signaling (Sheldahl et al., 2003). Activation of small GTPases, such as Rho and Rac, occurs downstream of DEP. These small GTPases activate JNK to regulate cellular polarity (Boutros et al., 1998; Yamanaka et al., 2002) (Figure 1-5). There are 3 subtypes of JNK in mammals: Jnk1, 2 and 3. Jnk1 is required for embryonic eyelid closure (Weston et al., 2003). Mice lacking Jnk1 and Jnk2 die during embryonic development because of a defect in neural tube closure (Kuan et al., 1999). These observations indicate that the JNK pathway regulates migration of epithelial and neuroepithelial cells. PDZ domain of Dvl is bound by PCP factors such as Dvl associated activator of morphogenesis 1 (DAAM1), Strabismus, Prickle, Prader-Willi/Angelman region – 1 (PAR1), and Diego (Wallingford and Habas, 2005).

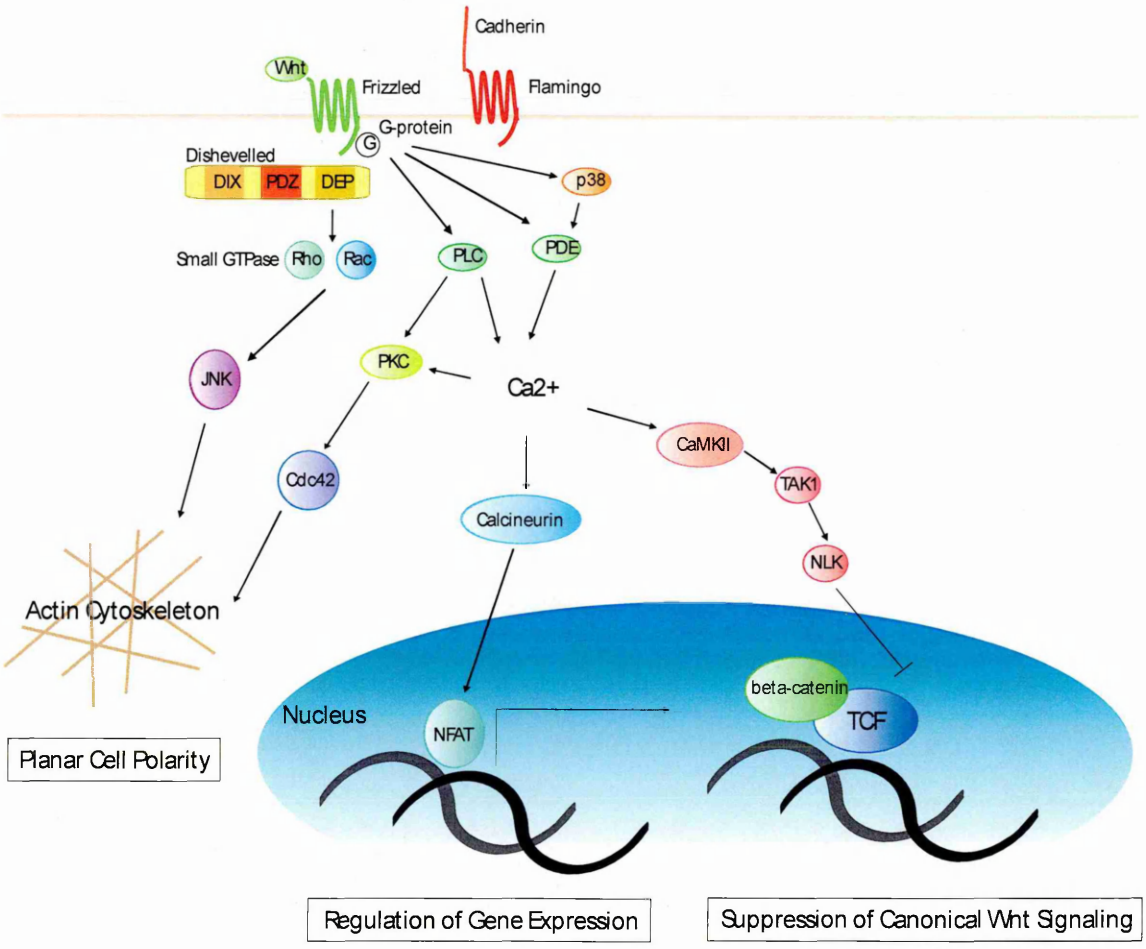

Figure 1-5 Non-canonical Wnt signaling

The activation of Fz by Wnt ligand is mediated by Dishevelled or heterotrimeric G-proteins. The signaling can be separated in 3 branches. Planar Cell Polarity is mediated by small GTPase (Rho and Rac), JNK and Cdc42 which is activated by PKC. NFAT transcriptional factor is activated by Ca2+-Calcineurin pathway to regulate gene expression. Calcium induced CaMKII-TAK1-NLK pathway suppresses canonical Wnt signaling by inhibiting β-catenin dependent transcription (Semenov et al., 2007). (Modified from Sugimura & Li, Birth Defect Research Part C, 90: 243-256, 2010)

1.6.7 Calcium mediated pathways

1.6.7.1 PKC-Cdc42 pathway

As a consequence of Frizzled activation by Wnt ligand, Frizzled mediates activation of heterotrimeric G-proteins. Activated G-proteins regulate phospholipase

C (PLC), phosphor di esterase (PDE) and p38 (Ahumada et al., 2002). After activation of these components, calcium ion relays signaling via downstream pathways as one of the secondary messengers (Kohn and Moon, 2005; Ma and Wang, 2007). Increase of intracellular calcium ion activates protein kinase C (PKC) (Sheldahl et al., 2003). Then, it regulates small GTPase Cdc42 (Schlessinger et al., 2007). Small GTPase Cdc42 is the key effector of PCP to remodel actin cytoskeleton and shape polarity of cells (Habas et al., 2003; Schlessinger et al., 2009; Schlessinger et al., 2007). Cdc42 is activated at the leading edge of migrating cells and provides actin-driven protrusions of cells (Etienne-Manneville and Hall, 2001, 2003) (Table 1-5).

Table 1-5 Mediators and transcription factors of non-canonical Wnt signaling

Mediator	Function	Reference
Dvl	Polarity of cells	Sheldahl, 2003
JNK	Polarity of cells	Sheldahl, 2003
Cdc42	Migration of cells	Etienne-Manneville,2001
CK1α	Suppress NFAT	Dejmek, 2006
Calcineurin	Angiogenesis	Courtwright, 2009
CaMKII	Bone formation	Takada,2007
	Inhibit canonical Wnt	Ishitani,2003
TAK1	Antagonize canonical Wnt signaling	Ishitani,2003
NLK	Antagonize canonical Wnt signaling	Ishitani,2003
Transcription factor	Function	Reference
NFAT	T-cell activation	Macian,2005
	Bone formation with Osterix	Koga,2005
	Inhibit canonical Wnt signaling	Saneyoshi,2002

1.6.7.2 CaMKII-TAK1-NLK pathway

The increase of calcium ion concentration in cytosol activates calcium/calmodulin-dependent kinase II (CamKII). CaMKII induces activation of Transforming growth factor-β-activated kinase 1 (TAK1) and Nemo-like kinase (NLK) kinases in Mitogen-activated protein kinase (MAPK) pathway (Ishitani et al.,

2003). CaMKII is involved in pathogenesis in the heart and brain (Wang et al., 2005a; Zhang et al., 2003b) (Table 1-5).

1.6.7.3 Calcineurin – NFAT pathway

Another branch of the calcium ion pathway is calmodulin-mediated activation of calcineurin (Saneyoshi et al., 2002). Calcineurin is a protein phosphatase known to activate NFAT transcription factor as described below.

1.6.8 Transcription factors

1.6.8.1 NFAT

NFAT alters cellular behavior through regulation of gene expression. The activated NFAT translocates to nucleus to regulate multiple processes such as T-cell proliferation and differentiation (Macian, 2005). The Calcineurin-NFAT pathway is one of the major non-canonical Wnt signaling pathways in vertebrates, and its deregulation leads to several diseases. In Down's syndrome, for example, the Down syndrome critical region gene 1 (DSCR1) and Dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYR K1A) genes are not only located in trisomy chromosome 21, but also their expression is increased, suggesting a role of these genes in Down's syndrome (Fotaki et al., 2002; Fuentes et al., 2000). Crabtree and colleagues found that Calcineurin and NFATc2 and NFATc4 double knockout mice had similar phenotypes as human Down's syndrome, such as defects in placenta, and in cardiovascular, neurological, gastrointestinal, skeletal, immune, and genitourinary systems (Arron et al., 2006). Mechanistically, increased expression of DSCR1 and DYRK1A synergistically prevent nuclear occupancy of NFAT transcriptional factors. In addition, NFAT is required for bone formation through

regulation of osteoblasts. Takayanagi and colleagues found that NFATc1 formed a complex with transcription factor Osterix in osteoblasts to induce bone formation. This observation provided important insight for management of osteoporosis as well as bone regeneration in osteogenic diseases (Koga et al., 2005) (Table 1-5).

1.6.9 Non-canonical Wnt signaling in stem cells

1.6.9.1 Hematopoietic stem cells

Recent studies revealed that HSCs contain two subpopulations: dormant (quiescent) and active, defined by function. The underlying mechanism that regulates these states is not clear. It has been proposed that active HSCs supply differentiated hematopoietic cells and support homeostasis. When active HSCs are depleted, such as by injury or disease, dormant HSCs function as a reserve pool to replenish active HSCs (Benveniste et al.; Elmslie et al., 1995; Haug et al., 2008; Wilson et al., 2008). A recent study by Morrison and colleagues knocked out Stem Cell Factor (SCF) in the perivascular cells and showed more than 90% loss of HSCs. However, the remaining HSCs still had 50% functional capacity (Ding et al., 2012). This suggests that the 10% remaining HSCs are a reserve population and are kept in quiescence and not affected by loss of SCF. But it is not known how this reserve pool of HSCs is maintained.

The function of canonical Wnt signaling is well-characterized in HSCs. β-catenin mediated transcription regulation promotes proliferation of HSCs. However, the role of non-canonical Wnt signaling in HSC development and maintenance remains largely unknown.

A recent report showed that knockout of Fz5 disrupted development of yolk sac and placenta, which are both primary sites for HSC development (Ishikawa et al., 2001). Another report showed that non-canonical Wnt signaling via Wnt11 induced hematopoietic cell fate from embryonic stem cells (Vijayaragavan et al., 2009). But the downstream event of non-canonical Wnt signaling that induces HSC development is not known. Given that NFAT has been shown to regulate HSC lineage (Muller et al., 2009), NFAT may be a link in this regard.

Wnt4 is one of the non-canonical Wnt signaling molecules that regulate HSCs (Louis et al., 2008). Wnt4 in HSCs activates genes required for cell maintenance such as *Cxcr4, Meis1, Pten, Ccnd2, Foxo1, Foxo4, Hoxa9, Hoxa10* and *Hoxb4*. Wnt4 inhibits cell-cycle through *Cdkn1b, Cdkn1c, Cdkn2d, Mxd1* and *Rbl2*. Wnt4 deficient mice showed low frequency of HSCs in BM, which raised the question whether non-canonical Wnt signaling is required to maintain HSCs in BM. A recent study by Bodine and colleagues provided evidence to support this idea (Nemeth et al., 2007). The researchers cultured HSCs in the presence of non-canonical Wnt5a protein. Wnt5a increased short- and long-term HSC repopulation by maintaining HSCs in the quiescent G0 phase. Also, Wnt5a inhibited Wnt3a-mediated canonical Wnt signaling in HSCs. However, this study was conducted only *in vitro* HSC culture, and *in vivo* function of non-canonical Wnt ligand remains unknown. In addition, the responsible receptor for non-canonical Wnt in HSCs is not clear.

Other reports have indicated that non-canonical Wnt signaling is required to maintain quiescent HSCs. In mice, deletion of Rac2 (a component of non-canonical Wnt signaling) led to enhanced mobilization of HSCs from BM which suggested activation of HSCs (Yang et al., 2001). The phenotype was associated with defective

cell adhesion and increased motility. In Rac2 deficient HSCs, Cdc42 (another component of non-canonical Wnt signaling) was upregulated as a compensation, which was associated with an increase in HSC motility. Conditional deletion of Cdc42 in HSCs showed a decrease of quiescent G0 phase and a defect of cell adhesion, resulting in impaired interaction between HSC and its endosteal niche where HSCs are kept in quiescence. Cdc42 deficient HSCs revealed significantly decreased expression of β1-integrin and N-cadherin, which may explain the loss of HSC adhesion with the HSC niche. Also Cdc42 deficient HSCs showed a significantly decreased p21 level and increased c-Myc level which would activate HSC proliferation (Yang et al., 2007). These reports suggest that HSCs are maintained in quiescence by non-canonical Wnt signaling; in contrast, canonical Wnt signaling activates HSCs (Table 1-6). My study has revealed *in vivo* function of non-canonical Wnt signaling for HSCs by investigating receptors and co-receptors of the signaling, Frizzled8 (Fz8) and Flamingo/Celsr2.

1.6.9.2 Skin stem cells

Skin stem cells are located in the bulge of hair follicles and are regulated by canonical Wnt signaling. Skin stem cells are activated by β-catenin-TCF mediated regulation of transcription, including activation of CyclinD1 and c-Myc, and inhibition of Bmp4 and E-cadherin (Alonso and Fuchs, 2003a, b). The function of non-canonical Wnt signaling was unknown until recently.

One of the main events downstream of non-canonical Wnt signaling in mammals is the regulation of NFATc1. A recent study by Fuchs and colleagues showed that NFATc1 was required to maintain quiescence of skin stem cells in hair

follicle (Horsley et al., 2008). NFATc1 expression in skin stem cells was activated by BMP signaling, which repressed Cdk4 expression. Repression of Cdk4 led to inhibition of cell-cycle progress which maintained skin stem cells in quiescence. (Table 1-6).

1.6.9.3 MSCs

MSCs (MSCs) in BM can self-renew and differentiate to other stromal components such as chondrocytes, adipocytes and osteoblastic cells (Uccelli et al., 2008) (Friedenstein et al., 1974; Friedenstein et al., 1976; Friedenstein et al., 1978). Wnt signaling plays a key role in regulation of MSCs, and its contribution has been studied in both canonical and non-canonical Wnt signaling. Tuan and colleagues showed that Wnt3a canonical signaling activated MSCs. Wnt5a non-canonical signaling maintained MSCs and induced osteogenesis *ex vivo* (Baksh and Tuan, 2007). Because MSCs are exposed by dynamic tension *in vivo* to form tendon or ligament tissues, researchers asked whether the same principle applied to *in vitro* culture. They observed regulation of Wnt gene expression with cyclic stimulation of tension, which suggested involvement of Wnt signaling during tendon formation from MSCs (Kuo and Tuan, 2008). Rubin and colleagues showed that mechanical stimulation of MSCs activated both β-catenin and NFATc1 through inhibition of GSK3β. This resulted in enhancing osteogenesis and inhibiting adipogenesis from MSCs. Molecular mechanisms underlying the observation are not yet known; however, the researchers indicated that induction of Cox2 by NFATc1 may regulate osteogenic or adipogenic differentiation of MSCs (Sen et al., 2009). Recent reports have suggested that MSCs and their progenies regulate HSCs (Arai et al., 2004;

Calvi et al., 2003; Mendez-Ferrer et al.; Naveiras et al., 2009; Nilsson et al., 2001; Sugiyama et al., 2006; Xie et al., 2009; Zhang et al., 2003a). Non-canonical Wnt signaling may be involved in the regulation of the interaction between MSCs and HSCs (Table 1-6).

Table 1-6 Stem cell and non-canonical Wnt signaling

Stem cell	Gene	Function	Reference
Hematopoietic	Wnt5a	Maintain quiescence	Nemeth,2007
	Wnt4	Maintain quiescence	Louis,2008
	Wnt11	Induce hematopoietic lineage from ES cells	Vijayaragavan,2009
	Fzd5	Yolk sac and placenta development	Ishikawa,2001
	Rac2	Regulate mobilization	Yang,2001
	Cdc42	Maintain quiescence	Yang,2007
		Inhibit cell-cycle	
		Regulate mobilization	
Skin	NFATc1	Maintain quiescence	Horsley,2008
Mesenchymal	Wnt5a	Maintain stem cells	Baksh,2007
		Induce osteogenesis	
		Induce tendogenesis	Kuo,2008
	NFATc1	Induce osteogenesis	Sen,2009
Neural	Wnt5a	Regulate dopaminergic differentiation	Parish,2008

1.6.9.4 Intestinal stem cells

Both the location and signaling regulation of intestinal stem cells (ISCs) have been a focus of study in gastroenterology (Scoville et al., 2008). The contribution of canonical Wnt signaling has been established in the activation of ISCs (He et al.,

2007; He et al., 2004a; Sancho et al., 2004); however, the function of non-canonical Wnt signaling is not clear. Clevers and colleagues reported that deletion of Frizzled5 (Fz5) changed the distribution of Paneth cell location in intestinal crypts (van Es et al., 2005). However, it is not understood whether non-canonical Wnt signaling contributes to this phenotype or whether this phenotype has significance in ISCs.

1.6.10 Non-canonical Wnt signaling in disease

Canonical Wnt signaling has been well characterized as one of the most important contributors to tumorigenesis (Reya and Clevers, 2005; Reya et al., 2001), and it has been implicated in over 40 types of solid tumors (Giles et al., 2003). Secretion of Wnt antagonists (such as DKK-1, Wnt inhibitory factor WIF, and sFRPs) is often downregulated in cancer. Many cancer cells show accumulation and nuclear localization of β-catenin. Since non-canonical Wnt signaling regulates stem cells, it may also play an important role in cancer. As non-canonical Wnt signaling maintains quiescence of stem cells and inhibits canonical Wnt signaling, it has been considered primarily a tumor suppressor. For example, mouse mammary cell transformation by an anti-sense Wnt5a mimicked canonical Wnt1-mediated transformation (Olson and Gibo, 1998). For another example, ectopic expression of Wnt5a in uro-epithelial cancer reverted tumorigenesis (Olson et al., 1997). However, other studies have indicated a different role for Wnt5a in tumorigenesis. Wnt5a was found unexpectedly to be overexpressed in cancers of lung, breast and prostate (Iozzo et al., 1995; Lejeune et al., 1995). More recently, it was reported that Wnt5a may enhance motility of malignant cells and tumor invasion such as in breast cancer, melanoma and gastric cancer (Kurayoshi et al., 2006; Pukrop et al., 2006). Trent and colleagues overexpressed Wnt5a in melanoma cells to determine its

function in tumor progression (Weeraratna et al., 2002). They found that Wnt5a increased motility and invasion of melanoma cells. To analyze further, they disrupted Fz5, a receptor of Wnt5a, by functional antibody. Disruption of Fz5 reduced melanoma invasion associated with inhibition of PKC activation. From Wnt5a staining of human melanoma tissues, the researchers found that a strong expression of Wnt5a correlated with a higher grade of tumor.

Gastric cancer is the fourth most common malignancy in the world (Parkin, 2001). To examine the relationship between aggressiveness of gastric cancer and the expression of Wnt5a, Kikuchi and colleagues studied 237 human gastric cancer cases (Kurayoshi et al., 2006). Expression of Wnt5a was observed in 30% of the cases. The intensity of Wnt5a expression correlated with advanced disease and poor prognosis. Also, cytosolic or nuclear accumulation of β-catenin was identified in 21% of the cases. However, Wnt5a and β-catenin were rarely expressed in the same gastric cancer cells, which implies that Wnt5a and β-catenin express in a mutually exclusive manner in gastric cancer, or in different stages of cancer. Whereas the contribution of β-catenin to tumorigenesis has been well characterized, the function of Wnt5a in gastric cancer is not known. When Wnt5a was knocked down in gastric cancer cells, cell migration activity was suppressed. Wnt5a activated both focal adhesion kinase and small GTP-binding protein Rac, which both play a role in cell migration. Since non-canonical Wnt signaling regulates cell motility, it may be that Wnt5a also enhances invasion of cancer in its later stages.

Deregulation of non-canonical Wnt signaling has been observed in hematologic malignancies. Using a Wnt5a null mice model, Jones and colleagues showed expansion and hyperproliferation of myeloid and B lymphocytes leading to

myeloid leukemia and B-cell lymphomas. (Liang et al., 2003). Wnt5a suppressed the expression of Cyclin D1 through the calcium pathway of non-canonical Wnt signaling and negatively regulated B-cell proliferation. To determine whether Wnt5a expression level is modulated in leukemia patients, researchers have studied the DNA methylation status of a Wnt5a promoter in acute lymphoid leukemia patients (Roman-Gomez et al., 2007). 43% of patients showed hypermethylation of a Wnt5a promoter, suggesting that Wnt5a was inactivated in those patients. Promoter hypermethylation of genes inhibiting canonical Wnt signaling (including Wnt5a) correlated with decreased rates of disease-free and overall survival (Martin et al., 2008). This indicates that non-canonical Wnt signaling suppressed leukemogenesis. In contrast, another recent study with chronic lymphoid leukemia (CLL) patients showed that Wnt5a increased viability of leukemia cells through Receptor-tyrosine-kinase-like orphan receptor1 (ROR1) and NF-κB induction (Fukuda et al., 2008). However, *in vivo* function of Wnt5a in CLL was not elucidated.

Wnt5a plays a role in tumor suppression, part of which may be via downregulating canonical Wnt signaling. In contrast, Wnt5a also promotes malignancy by enhancing invasion of cancer cells in the later stages of cancer (Figure 1-6). This discrepancy may arise from multiple factors regulating Wnt5a as well as from observations at different stages of tumor progression.

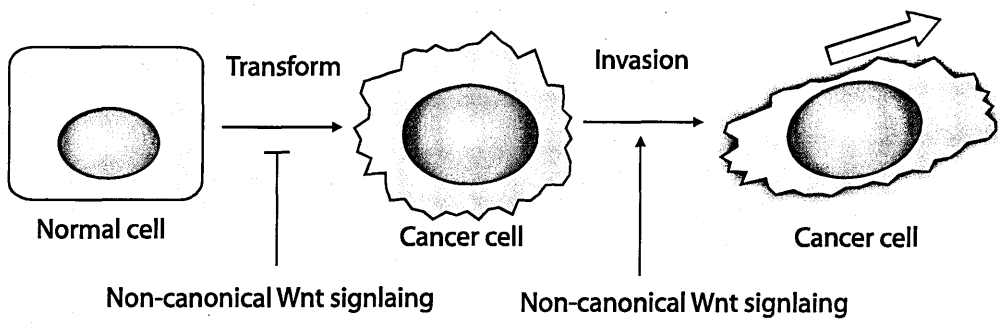

Figure 1-6 Dual function of non-canonical Wnt signaling in cancer

Non-canonical Wnt signaling suppresses transformation of cells. However, in late stage cancer, non-canonical Wnt signaling enhances invasion of cancer cells which leads to metastasis of cancer. (Modified from Sugimura & Li, Birth Defect Research Part C, 90: 243-256, 2010)

Wnt5a is a main focus of study of non-canonical Wnt signaling in cancer. However, there are few reports regarding receptors and cytoplasmic mediators of non-canonical Wnt signaling in cancer. A recent study by DeGregori shed light on Frizzled8 (Fz8), a receptor of Wnt signaling (Gregory et al., 2010) . Using shRNA library, researchers screened genes which, when inhibited, made chronic myeloid leukemia (CML) cells sensitive to imatinib, an inhibitor of Bcr-Abl tyrosine kinase. Although imatinib is a highly effective treatment for patients with CML, it is rarely curative because of relapse, even in patients that initially show a complete response (Deininger et al., 2005; Rousselot et al., 2007). This underscores the need to identify targets that will cooperatively treat CML with imatinib. From the gene screening, Fz8 was identified by three different shRNAs. Ectopic expression of Fz8 was capable of activating β-catenin-dependent canonical Wnt signaling when co-expressed with co-receptor LRP6 (Liu et al., 2005). However, studies performed in Xenopus implicated that Fz8 function in β-catenin is independent of Wnt signaling (Wallingford et al., 2001). Through screening in CML cells, researchers identified isoforms of both CaMKII

and PKC, components of non-canonical Wnt signaling. They tested whether Fz8 mediates non-canonical Wnt signaling in CML cell line K562 by knocking down Fz8. K562 was infected with NFAT reporter construct to measure activity of non-canonical Wnt signaling. Calcium ionophore ionomycin rescued downregulation of NFAT activity when Fz8 was knocked down, suggesting that Fz8 mediated non-canonical Wnt signaling through the calcium pathway to regulate NFAT activity. Furthermore, the researchers showed that NFATc1 protected CML cells from imatinib-induced cell death via induction of IL-4 expression. They indicated that Fz8-mediated non-canonical Wnt signaling was responsible for imatinib resistance in CML.

There is growing body of evidence that interaction between tumor cells and the stromal components is crucial for malignant progression (Raaijmakers et al., 2010). Binder and colleagues co-cultured breast cancer cell lines with macrophages, which led to upregulation of Wnt5a (Pukrop et al., 2006). Wnt5a derived from macrophages induced invasion of breast cancer cells. The report suggested that Wnt5a-mediated non-canonical Wnt signaling contributed to enhanced malignancy through interaction with neighboring macrophages. Surprisingly, DKK-1, a well-characterized inhibitor of canonical Wnt signaling, antagonized Wnt5a-induced invasion of breast cancer cells without affecting β-catenin signaling. Though further study is required, this indicates that DKK-1 may regulate Wnt5a-mediated non-canonical Wnt signaling (Table 1-7).

Table 1-7 Diseases and non-canonical Wnt signaling

Cancer	Genes	
Hematologic malignancy		
B-cell lymphoma	Wnt5a	B-cell lymphoma in null mice
Acute lymphoid leukemia	Wnt5a	Hypermethylation of Wnt5a promoter in patients
Chronic lymphoid leukemia	Wnt5a	Increased viability of leukemia cells
Chronic myeloid leukemia	Fzd8	Resistance to imatinib
	NFAT	Resistance to imatinib
Solid tumor		
Mammary cell transformation	Wnt5a	Antisense Wnt5a mimcs Wnt1-mediated transformation
Uro-epithelial cancer	Wnt5a	Reverted tumorigenesis
Lung cancer	Wnt5a	Upregulated expression
Breast cancer	Wnt5a	Upregulated expression, macrophage-induced invasion
Prostate cancer	Wnt5a	Upregulated expression
Melanoma	Wnt5a	Upregulated expression, increased motility
Gastric cancer	Wnt5a	Increased motility
Other diseases		
XY intersex	Wnt4	Overexpression inhibited testicular development
Down's syndrome	Calcineurin	Knockout mice showed phenocopy of human Down's
	NFATc	Knockout mice showed phenocopy of human Down's
Cardiac hypertrophy	Fzd2	Expressed during hypertrophy
	NFAT	Pathological hypertrophy
	CaMKII	Activated in hypertrophy
Alzheimer's disease	CaMKII	CaMKII containing neurons were lost selectively

1.7 My Study in Context of the HSC Field

In the HSC field, it has not yet been made clear how quiescent and active HSCs are regulated in the niche. Although Wnt signaling is one of the best studied signaling in the field, the functional role of non-canonical Wnt signaling has not been identified *in vivo*. In addition, it has been controversial where quiescent HSCs are located in the BM niche. In my study, I have investigated the functional role of non-canonical Wnt signaling by studying receptors Flamingo/Celsr2 (Fmi) and Frizzled8 (Fz8) expressed in quiescent HSCs.

Furthermore, I have analyzed the niche components which facilitate non-canonical Wnt signaling.

1.8 Hypothesis

Non-canonical Wnt signaling via its receptors in quiescent HSCs functions to maintain HSCs.

1.9 Strategy of My Study

First, microarray analysis of quiescent HSC was done to pick up non-canonical Wnt genes. Then, shRNA of each gene was done to see the phenotype in HSCs, which was further confirmed by knockout mouse model. Finally, I analyzed the cellular mechanism and molecular mechanism of non-canonical Wnt signaling function in HSCs (Figure1-7).

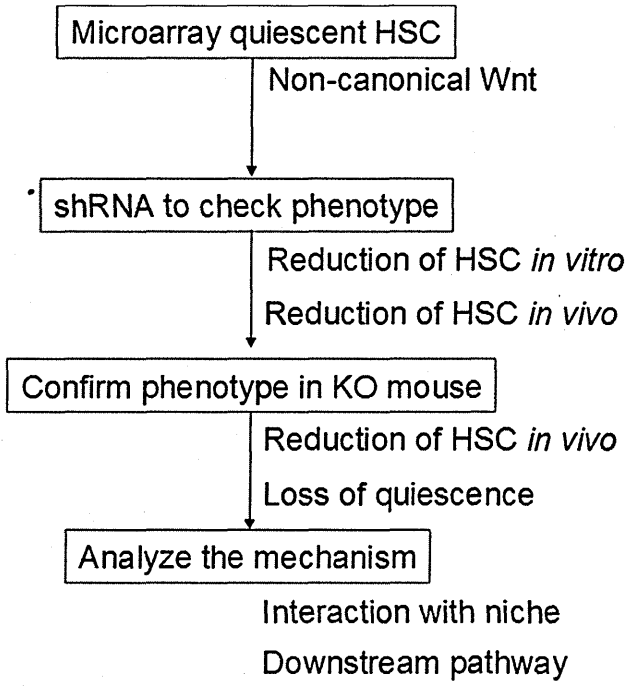

Figure 1-7 Strategy of my study

Chapter 2. Materials and Methods

2.1 Animals

Both *Flamingo/Celsr2*$^{+/-}$ (Tissir et al., 2010) and *Frizzled8*$^{+/-}$ (Ye et al., 2011) mice were obtained from the Jackson Laboratory (Celsr2tm1 and Fzd8tm respectively) and bred to obtain homozygous mice. Some heterozygous female mice were infertile from uterus atresia, and I observed less number of homozygous. H2B-GFP mice and SCL-tTA mice were also obtained from Jackson labs (TRE-mCMV-H2B-GFP and Tal1-tTA respectively). Nestin-GFP and Col2.3-GFP mice were kindly provided by Grigori Enikolopov (Cold Spring Harbor Laboratory) and David Rowe (University of Connecticut Health Center). CD31-GFP mice were generated in our lab, in which we used the CD31-GFP ESCs kindly provided by Dr. Virginia Bautch (University of North Carolina). TOP-GAL mice were kindly provided by Dr. Elaine Fuchs (Rockefeller University). Axin2-d2EGFP mice were kindly provided by Dr. Frank Costantini (Columbia University). All of the mice in this study were kept in C57BL/6J background. All mice used in this study were housed in the animal facility at the Stowers Institute for Medical Research (SIMR) and handled according to SIMR and National Institute of Health (NIH) guidelines. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of SIMR.

2.2 Flow cytometry

For phenotype analysis, hematopoietic cells were harvested from BM (femur and tibia), spleen, and peripheral blood. Red blood cells were lysed using a 0.16M ammonium chloride solution. For cell surface phenotyping, a lineage cocktail (Lin, PE-Cy5) was used including CD3, CD4, CD8, Mac-1, Gr1, B220, IgM, and Ter119

(eBioscience,). Monoclonal antibodies against Sca-1, c-Kit, Flk2, CD34, CD150, GRP78 (Sigma), CD45.1, and CD45.2 were also used where indicated. Biotinylated monoclonal N-cadherin antibody (AbD13077, Toshio Suda, Keio University) and streptavidin-PE-Cy7 were used for sorting bone cells. Cell sorting and analysis were performed using a MoFlo (Dako), InFlux Cell Sorter (BD Biosciences) and/or CyAn ADP (Dako). Data analysis was performed using FlowJo software.

2.3 Cell-cycle analysis

Cell-cycle analysis of HSCs was conducted with BD PhamingenTM FITC Mouse Anti-Human Ki67 Set according to manufacturer's instruction. The cells were further incubated with 0.1ug/uL DAPI for 30min at room temperature followed by flow cytometric analysis with InFlux Cell Sorter (BD Biosciences).

2.4 HSC culture

HSC expansion media (ST + insulin media) consisted of StemSpan SFEM media (Stem Cell Technologies) supplemented with 10 μg/ml heparin (Sigma), 0.5× Penicillin/Streptomycin (Sigma), 10 ng/ml recombinant mouse (rm) Stem cell Factor (SCF) (Biovision) and 20 ng/ml rm (Thrombopoietin) Tpo (Cell Sciences) (Perry et al., 2011b). BM cells were harvested from C57BL/6J (CD45.2) mice and made into a single cell suspension by gently passing through a 22g needle 3-5 times. Mouse Wnt3a (100 ng/uL, Millipore), mouse Wnt5a (500 ng/uL, R&D), and human Wnt7b (100 ng/uL, Novus Biologicals) were added in culture as indicated. The concentration of Wnt ligands used was according to a previous report (Nemeth et al., 2007). NFAT inhibitor (11R-VIVIT, Cell-permeable, Calbiochem) was used in the HSC expansion media (1uM).

2.5 Lentivirus construction

The pSicoR-EF1α promoter-IRES-EGFP lentiviral construct was kindly provided by Dr. Ting Xie (SIMR). CA-NFAT from Addgene (distributed by Anjana Rao at Immune Disease Institute) and shRNA for Flamingo and Frizzled8 were cloned into SmaI site of the construct. The sequences of Flamingo and Frizzled8 shRNA used were from a previous report (Shima et al., 2004) and from RNAi Codex (http://cancan.cshl.edu/cgi-bin/Codex/Codex.cgi).

Flamingo shRNA 1 Fwd:
AGCGCCGTGCATGCGCACACGAAGATTAGTGAAGCCACAGATGTAATCT
TCGTGTGCGCATGCACGA
Flamingo shRNA 1 Rev:
GGCATCGTGCATGCGCACACGAAGATTACATCTGTGGCTTCACTAATCTT
CGTGTGCGCATGCACGG
Flamingo shRNA 2 Fwd:
AGCGCGCTGGCTCTCTGTCTATGATATAGTGAAGCCACAGATGTATATCA
TAGACAGAGAGCCAGCA
Flamingo shRNA 2 Rev:
GGCATGCTGGCTCTCTGTCTATGATATACATCTGTGGCTTCACTATATCAT
AGACAGAGAGCCAGCG
Frizzled8 shRNA Fwd:
AGCGCCCGAATCCGTTCAGTCATCAATAGTGAAGCCACAGATGTATTGAT
GACTGAACGGATTCGGA
Frizzled8 shRNA Rev:
GGCATCCGAATCCGTTCAGTCATCAATACATCTGTGGCTTCACTATTGAT
GACTGAACGGATTCGGG
Scramble shRNA Fwd:
AGCGCCGTGCATGCGCACACGAAGATTAGTGAAGCCACAGATGTAATCT
TCGTGTGCGCATGCACGA
Scramble shRNA Rev:
GGCATCGTGCATGCGCACACGAAGATTACATCTGTGGCTTCACTAATCTT
CGTGTGCGCATGCACGG

2.6 Lentivirus infection

Mice were treated with 150 ug/g body weight of 5FU to activate and enrich for HSPCs (Miller and Eaves, 1997). 4 days later, BM was harvested and cultured

overnight in ST media and transduced by MagnetofectionTM using ViroMag R/L particles according to the manufacturer's protocol (OZ Biosciences).

2.7 Transplantation and Homing assay

Sorted 200 LSK cells and $2x10^5$ rescue BM cells were transplanted for Flamingo and Frizzled8 knockout model. Propidium iode staining was used to determine viability of sorted LSK. 50% (100) of the sorted LSK cells were alive. Transplantation experiments were conducted in the knockdown model using unsorted $3x10^5$ infected BM cells (CD45.2). The cells were transplanted into each lethally irradiated (10Gy) Ptprc (CD45.1) recipient. Homing assay was performed according to a previous report (Zhang et al., 2006). $5x10^6$ BM cells (labeled with CFDA, Invitrogen) were injected to each lethally irradiated (10Gy) Ptprc (CD45.1) recipient. 16 hours post injection, femur and spleen were collected to compare the number of CFDA+LSK prior to injection.

2.8 Repopulation assay

16 weeks post transplantation, peripheral blood was collected from submandibular vein. The hematopoietic repopulation was measured from donor-derived blood cells (CD45.2).

2.9 RNA-sequencing

The RNA-sequencing library was prepared from approximately 200 ng of total RNA [CD31-GFP$^+$ (VEGFR2$^+$CD45$^-$Ter119$^-$) cells, Nestin-GFP$^+$ (CD45$^-$CD31$^-$ Ter119$^-$) cells, N-cad$^+$OBs (CD45$^-$CD31$^-$Ter119$^-$) and mature OBs Col2.3-GFP$^+$ (CD45$^-$CD31$^-$Ter119$^-$)] for each sample using illumina TruSeq RNA Sample Prep Kit (Catalog #: FC-122-1001). The fragment size in the generated library ranged

from 220 to 500 bps with a peak at 280 bps. A total of 10 fmol library fragments were loaded to cBot to generate clusters, followed by sequencing on an Illumina HiSeq 2000 to produce 10-30 million paired-end 100 bp reads per sample. Reads were trimmed to 70bp due to quality, and aligned to mm9 with Tophat 1.3.1 (Trapnell et al., 2009) / Bowtie 0.12.7(Langmead et al., 2009), using the Ensembl 63 GTF file for gene models. Parameters were -g 1 --mate-inner-dist 200 --mate-std-dev 70 --segment-length 35 --segment-mismatches 2; this allowed for 4 mismatches per read (two per read half) and unique alignments only. Gene expression was quantitated using Cufflinks 1.0.3 (Trapnell et al., 2010).

2.10 Calcium level assay

BM cells were incubated in PBS/2%FBS containing Fluo-3 (Molecular Probes) according to the manufacture's instruction. Ionomycin (Sigma) was used as a positive control to measure intracellular Ca2+ level in LT-HSCs.

2.11 Mitochondrial activity assay

BM cells were incubated in PBS/2%FBS containing Mitotracker Green FM (Molecular Probes) according to the manufacture's instruction. The Mitotracker signal was measured by FITC channel in flow cytometry.

2.12 qRT-PCR

Primary HSCs or infected HSPCs were sorted by flow cytometry as described above. The cells were lysed, reverse transcribed, and pre-amplified by TaqMan® PreAmp Cells-to-CTTM Kit (Ambion) according to the manufacturer's instruction. TaqMan® gene expression assays (Applied Biosystems) were performed on triplicate samples using a 7900HT fast real-time PCR system (Applied Biosystems).

The primer sequences and TaqMan (Applied Biosystems) serial numbers are listed in Appendix.

2.13 Production of the recombinant anti-mouse N-cadherin Fab antibody

The phage display library, Human Combinatorial Antibody Libraries (HuCAL® Gold, AbD Serotech) was used and isolated recombinant Fab against mouse N-cadherin (AbD13077). In brief, HuCAL phage library was screened by biopanning using N-cadherin extracellular fragment-Fc fusion protein. After the first screening, the ELISA screening, clone sequencing, and QC ELISA with positive and negative control proteins were performed. Since N-cadherin binds not only to N-cadherin but also R- and OB-cadherins, R- and OB-cadherins-Fc fusion proteins were used for the negative controls. Seven Fab clones were obtained that react to N-cadherin but not to R- and OB-cadherin. These Fab clones were characterized for their availability for flow cytometry using NIH3T3 cell line and mN-cadherin-overexpressing Ba/F3 cell line. AbD13077 was useful for the flow cytometric analysis and cell sorting.

2.14 Immunostaining

Paraffin sections of bone were deparaffinized at 600C for 20min. Then, the sections were treated with 100% Xylene for 5min twice, 100% Ethanol for 5min, 95% Ethanol for 5min, 70% Ethanol for 5min, water rinse for 1min, followed by antigen retrieval with Citrate buffer at 900C for 10min. Blocking was done with Universal Blocking Reagent (BioGenex). The following antibodies were used: Flamingo (Celsr2) antibody (rabbit, 1:25, NLS1943, Novus Biologicals), Frizzled8

antibody (goat, 1:100, NB100-2439, Novus Biologicals), Osterix antibody (rabbit, 1:100, ab22552, abcam), NFAT antibody (mouse, 1:100, sc-7294, Santa Cruz), biotinylated monoclonal N-cadherin antibody AbD13077 (against mouse N-cad but fused with human-Fc, provided by Dr. Toshio Suda, Keio University), GFP antibody (chicken, 1:200, ab13970, abcam), pS552-β-catenin polyclonal antibody (rabbit, 1:200, Li lab). Secondary staining was done with donkey anti-rabbit AF546, donkey anti-mouse AF546, donkey anti-chicken DL488, donkey streptavidin-DL488, donkey streptavidin-AF594 and donkey anti-goat DL649 (Invitrogen). Each dilution was 1:200. For immunostaining of sorted cells, cells were sorted onto lysine-coated slides, fixed with chilled methanol for 10min followed by blocking and staining with primary antibody (Ema et al., 2006). For high-resolution 3D images, the Z-stack collected images from LSM 510 VIS Confocal Microscopy (Zeiss) were analyzed with Imaris software (Bitplane). For H2B-GFPhi LRC image, H2B-GFP mean fluorescence intensity (MFI) was >80,000. The population correlated with H2B-GFPhi in flow cytometry.

2.15 Isolation of endosteal and central marrow hematopoietic stem cells

Central marrow cells were isolated from femur and tibia. For endosteal BM cells, the edge of femur and rest of flushed bones were collected and ground, followed by digestion with collagenase I (3mg/ml) and dispase II (4mg/ml) at 370C for 20 min. in a horizontal shaker (Grassinger et al., 2010).

Chapter 3. Flamingo regulates non-canonical Wnt receptor Frizzled8 distribution in quiescent long-term HSCs

In this chapter, I will examine the expression of Flamingo/Celsr2 (Fmi) and Frizzled8 (Fz8) in HSC subpopulation. Then, I will test whether and how Fmi regulates distribution of Fz8 protein on the surface of HSCs.

Xi He previously reported expression of both *Celsr2/Flamingo (Fmi)* and *Frizzled (Fz) 8* in quiescent HSCs (Rhodamine^{lo} or Rh123^{lo}LSK) as detected by microarray analysis (Akashi et al., 2003). As these non-canonical Wnt receptors were highly expressed in quiescent HSCs, I hypothesized Fmi and Fz8 may regulate HSC maintenance. To further confirm Fmi expression in HSCs, I compared its expression level in long-term (LT)-HSCs (CD34$^-$Flk2$^-$LSK), short-term (ST)-HSCs (CD34$^+$Flk2$^-$LSK), and multipotent progenitors (MPPs) (CD34$^+$Flk2$^+$LSK) (Figure 3-1A-B) (Yang et al., 2005). qRT-PCR analysis revealed that Fmi mRNA level in LT-HSCs was 2-fold and 3-fold higher than in ST-HSCs and MPPs respectively (Figure 3-1C).

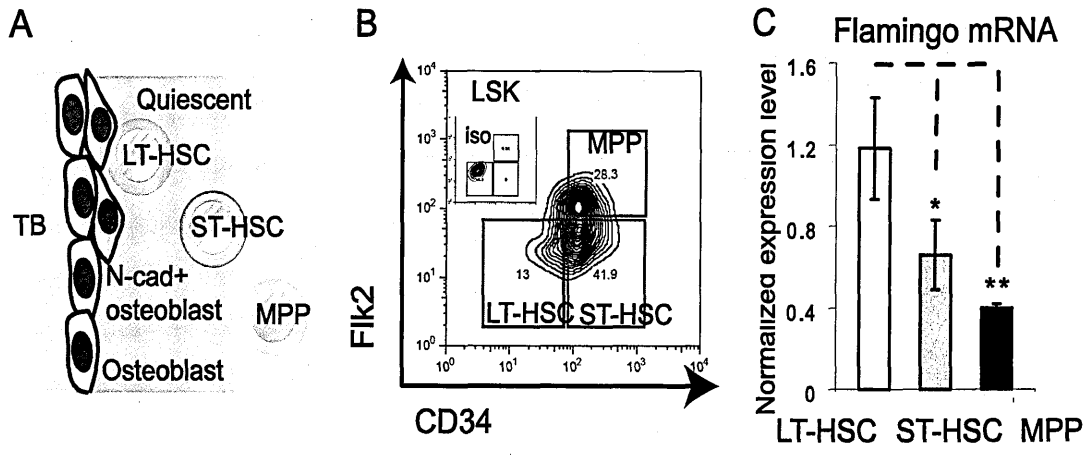

Figure 3-1 Expression of Fmi and Fz8 in LT-HSCs, ST-HSCs and MPPs

(A) LT-HSCs, ST-HSCs and MPPs with N-cad$^+$OBs in BM. (B) Gating LT-HSCs (CD34$^-$Flk2$^-$LSK), ST-HSCs (CD34$^+$Flk2$^-$LSK), and MPPs (CD34$^+$Flk2$^+$LSK). (C) qRT-PCR analysis of *Fmi* expression in sorted LT-HSCs, ST-HSCs, and MPPs in a setting of triplicates. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

I next examined Fmi and Fz expression in quiescent LT-HSCs that were identified using H2B-GFP label-retaining cells (LRCs) (Blanpain et al., 2004; Foudi et al., 2008; Tumbar et al., 2004; Wilson et al., 2008). H2B-GFP was expressed under the control of Doxycycline (Dox)-inducible SCL-promoter/enhancer -tTA, which is specific to hematopoietic stem and progenitor cells (HSPCs) (Koschmieder et al., 2005). The mice with SCL- tTA driven H2B-GFP were induced and then chased for 120 days by long-term Dox feeding, during which time H2B-GFP marking was lost from the majority of cycling HSCs (Figure 3-2A). I excluded H2B-GFP background signal derived from non-tTA induced animals (Challen and Goodell, 2008; Wilson et al., 2008) and set H2B-GFP gate at the high position (H2B-GFP^{hi}) (Figure 3-2B, see Figure 3-2C for unchased positive control).

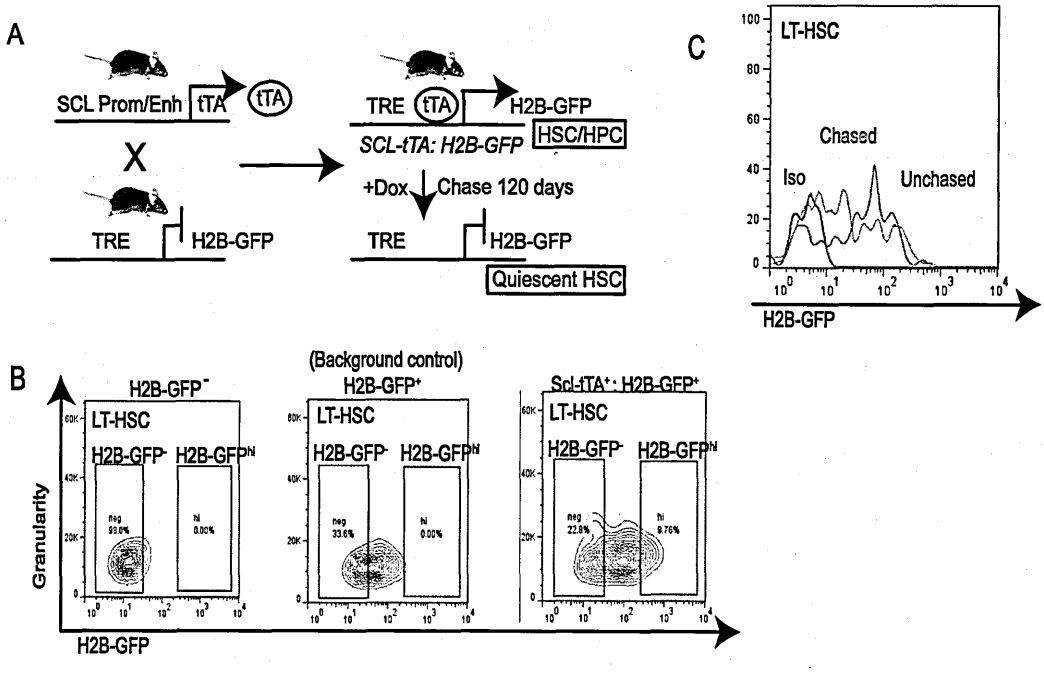

Figure 3-2 Labeling quiescent HSCs with H2B-GFP

(A) *SCL-tTA: H2B-GFP* mouse model. H2B-GFP was expressed under SCL-tTA and chased 120 days with Dox to wash out label in cycling cells. (B) Controls for H2B-GFP model. H2B-GFP$^-$ (left panel), Scl-tTA$^-$: H2B-GFP$^+$ (background control, middle panel) and Scl-tTA$^+$: H2B-GFP$^+$ (sample, right panel). (C) Controls for H2B-GFP model. Iso (H2B-GFP$^-$, red), chased (brown), and unchased (blue). (Modified from Sugimura et al., Cell 150, 351-365, 2012)

I confirmed that Scl-H2B-GFP^{hi}LSKs enriched LT-HSCs 5-fold and had fewer (4.8-fold) MPPs compared to Scl-H2B-GFP$^-$LSKs (Figure 3-3A). I further sorted Flk2$^-$LSK HSCs (to exclude MPPs) into H2B-GFP$^-$ (active) and H2B-GFP^{hi} (quiescent) subpopulations (Figure 3-3B). Consistent with a previous report (Wilson et al., 2008), I found that CD34 mRNA had a much higher expression in active HSCs than in quiescent HSCs (Figure 3-3C). Fmi expression was 12.5-fold higher in quiescent HSCs than in active HSCs (Figure 3-3D). Among Fzs, non-canonical Wnt receptor Fz8 showed significantly higher (3-fold) expression in quiescent HSCs than in active HSCs (Figure 3-3E-F).

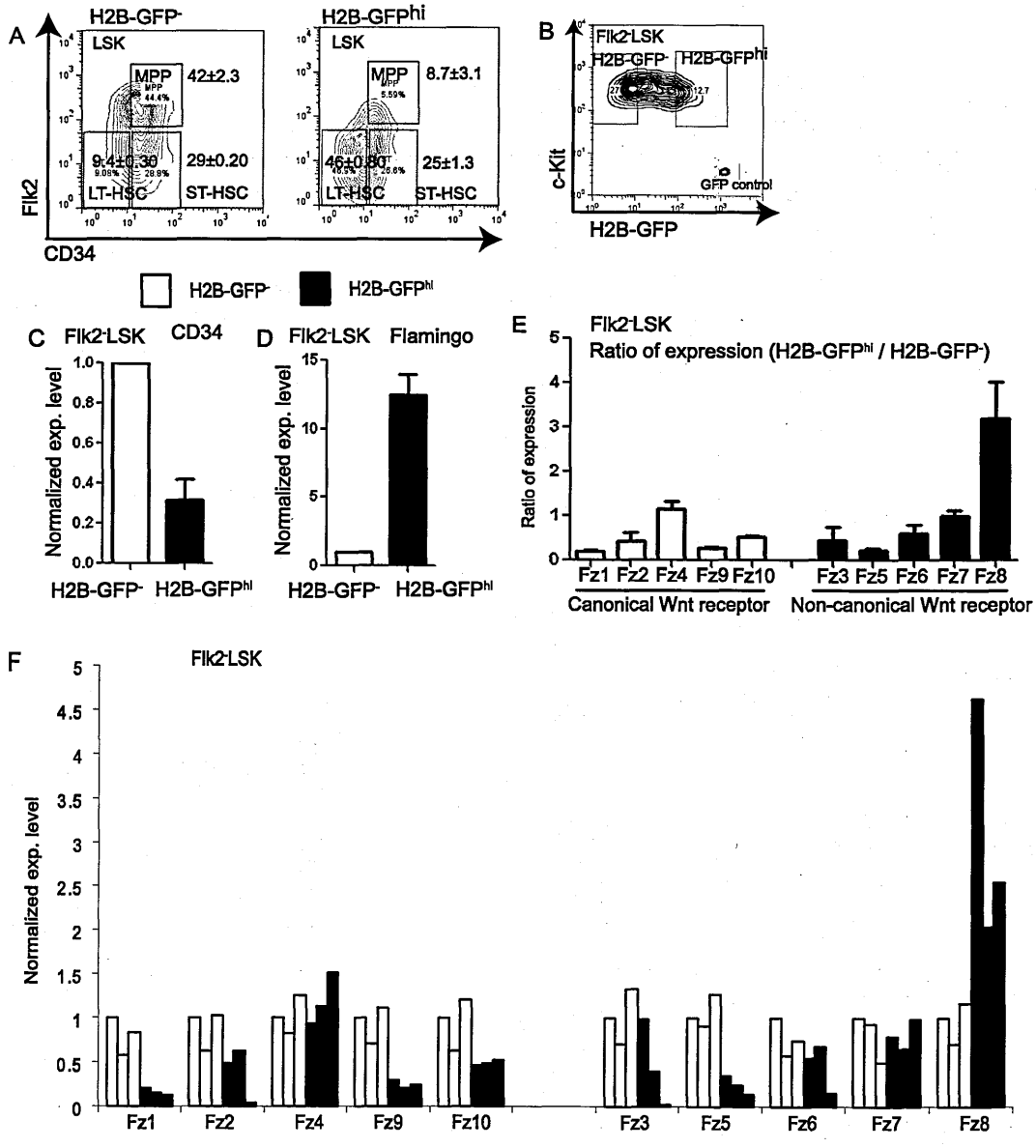

Figure 3-3 Expression of Fmi and Fzs in quiescent HSC and active HSC

(A) Percentage of LT-HSCs, ST-HSCs and MPPs in H2B-GFP$^-$LSK (left) and H2B-GFP^{hi}LSK (right). (B) Gating H2B-GFP HSCs (Flk2$^-$LSK). (C-E) qRT-PCR analysis of *CD34* (C), *Fmi* (D), *Fzs* (E) in H2B-GFP$^-$Flk2$^-$ LSK and H2B-GFP^{hi}Flk2$^-$LSK. (F) qRT-PCR of Fzs in H2B-GFP$^-$Flk2$^-$LSK (active HSC) and H2B-GFP^{hi}Flk2$^-$ LSK (quiescent HSC). (Modified from Sugimura et al., Cell 150, 351-365, 2012)

To confirm Fmi and Fz8 protein expression in LT-HSCs, I immunostained sorted LT-HSCs and found that both Fmi and Fz8 were expressed in LT-HSCs (Figure 3-4A). The expression of Fmi and Fz8 protein, as revealed by immunoassay, was detected in 18% of H2B-GFP$^-$ HSCs compared to 70% of H2B-GFP^{hi} HSCs

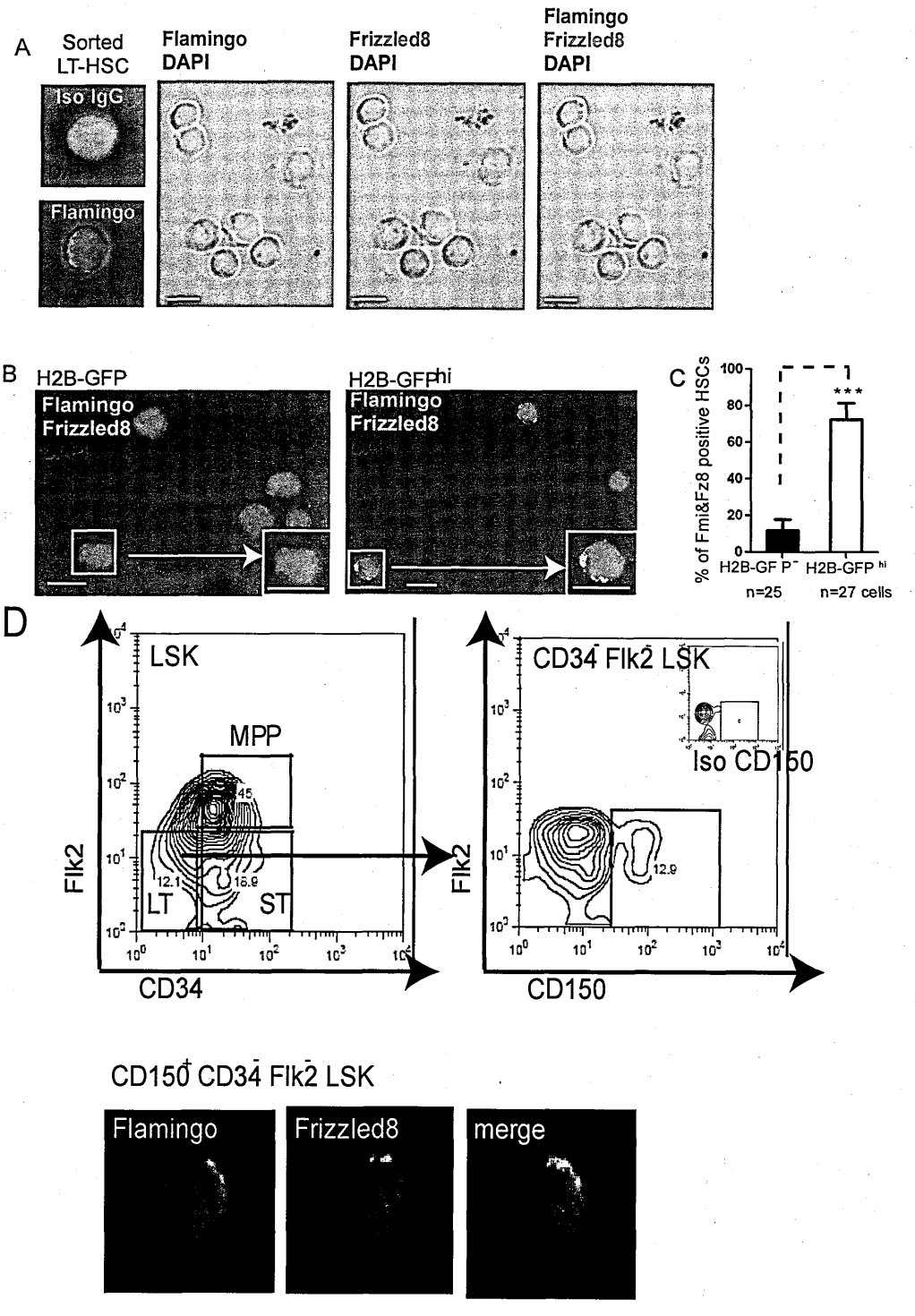

Figure 3-4 Expression of Fmi and Fz8 in HSC subpopulations

(A-B) Immunostaining Fmi and Fz8 in sorted LT-HSCs (A) and H2B-GFP HSCs (B). Endogenous H2B-GFP signal diminished by methanol fixation for further immunostaining. (C) Percentage of Fmi and Fz8 expressed in sorted H2B-GFP HSCs. (D) Fmi and Fz8 immunostaining of $CD150^{+}CD34^{-}Flk2$-LSK. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

(Figure 3-4B-C). I also detected the expression of Fmi and Fz8 in $CD150^{+}CD34^{-}$ $Flk2^{-}LSK$ HSCs (Figure 3-4D). I used a recently reported hypoxic-related HSC marker GRP78 (Miharada et al., 2011) and detected Fmi and Fz8 expression in $GRP78^{+}CD34^{-}LSK$ HSCs as well (Figure 3-5).

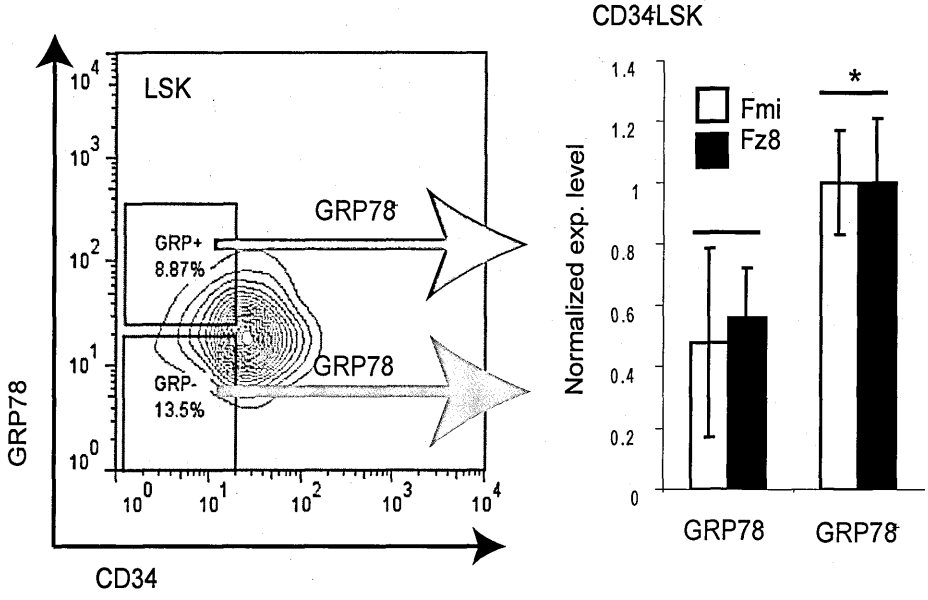

Figure 3-5 Expression of Fmi and Fz8 in HSC subpopulations

qRT-PCR for Fmi and Fz8 comparing $GRP78^{+}CD34^{-}LSK$ and GRP78-CD34-LSK. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

Fmi has been reported to determine the cellular distribution of Frizzled protein (Devenport and Fuchs, 2008; Tissir et al., 2010). I confirmed this by overexpressing Fmi and Fz8 fused with fluorescent proteins in 293T cells. I found that Fz8 distributed randomly when Fmi was not expressed; however, Fz8 was restricted to the site where Fmi was present (Figure 3-6A). To ascertain whether Fz8 distribution in HSCs was determined by Fmi, I used an *in vitro* culture system of HSCs with OP9, an osteoprogenitor cell line (Mahmood et al., 2011; Nakano et al., 1994). (Figure 3-6B). Immunostaining showed that Fmi was present at the interface

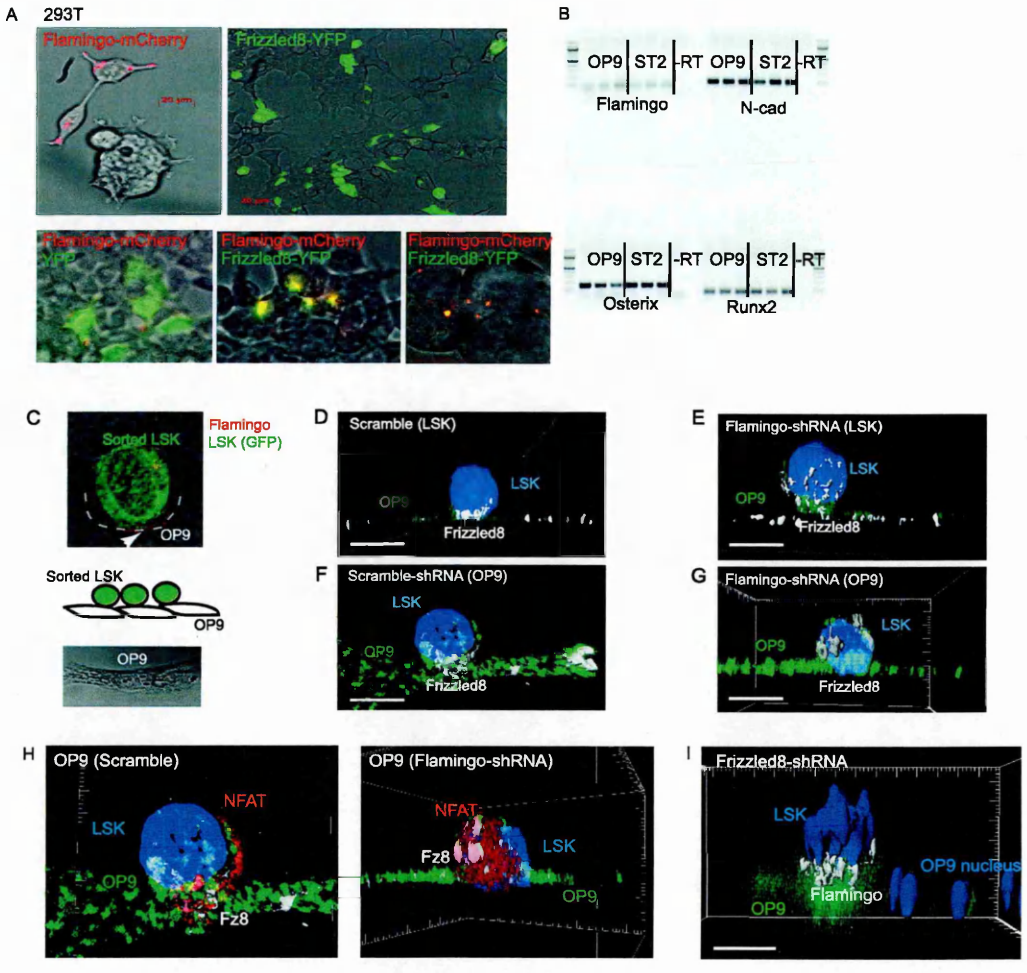

Figure 3-6 Fmi regulates Fz8 localization in HSC

(A) Fmi restricts Fz8 protein distribution in 293T cells. (B) OP9 osteoprogenitor cells express Fmi, N-cad, Osterix and Runx2. (C) Immunostaining Fmi in sorted LSK cells (GFP labeled) and OP9 osteoprogenitors. (D-G) 3D-images of Fz8 immunostaining of LSK cells on OP9. Scale bar is 5uM. (H) Fmi in OP9 also restricts Fz8 distribution at the interface between LSK and OP9, and affects nuclear vs. cytoplasmic localization of NFAT. (I) Fz8 does not restrict Fmi distribution at the interface between LSK and OP9. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

between sorted LSK (labeled with GFP) and OP9 cells (Figure 3-6C). When Fmi was knocked down, the distribution of Fz8 protein became random (Figure 3-6D-E). In addition, lack of Fmi in OP9 also resulted in random distribution of Fz8 in LSK cells (Figure 3-6F-H), suggesting that homophilic interaction between two Fmi located in adjacent HSC and OP cells functions to restrict Fz8 at the HSC-OP9 interface. In

contrast, knockdown of Fz8 in LSK did not affect Fmi protein distribution at the HSC-OP9 interface (Figure 3-6I). These results show that Fmi and Fz8 are both expressed in quiescent, long-term HSCs and that Fmi restricts Fz8 distribution at the interface between HSCs and osteoprogenitors *in vitro*.

Chapter 4. Flamingo and Frizzled8 co-localize predominantly at the interface between quiescent LT-HSCs and N-cad^{+}OBs in the TBR

In this chapter, I will examine where Fmi and Fz8 co-localized between quiescent HSCs and niche components.

I conducted immunostaining of Fmi and Fz8 with H2B-GFP^{hi} label-retaining quiescent HSCs and niche reporters with GFP. I next confirmed *in vitro* observation of the Fmi and Fz8 co-localization *in vivo*. Since HSCs tend to home to the TBR (Figure 4-1A-B) rather than the compact bone region (CBR) (Xie et al., 2009), I examined the distribution of Fmi-Fz8 co-expressing LT-HSCs in different bone regions, including the TBR and the CBR (Figure 4-1B). I sorted LT-HSCs (CD34^{-} Flk2^{-}LSK) and immunostained with anti-Fmi and anti-Fz8 antibodies (Figure 4-1C-D). I detected a majority (67%) of LT-HSCs derived from the TBR, but only 31% of those from the CBR expressed both Fmi and Fz8 (Figure 4-1E).

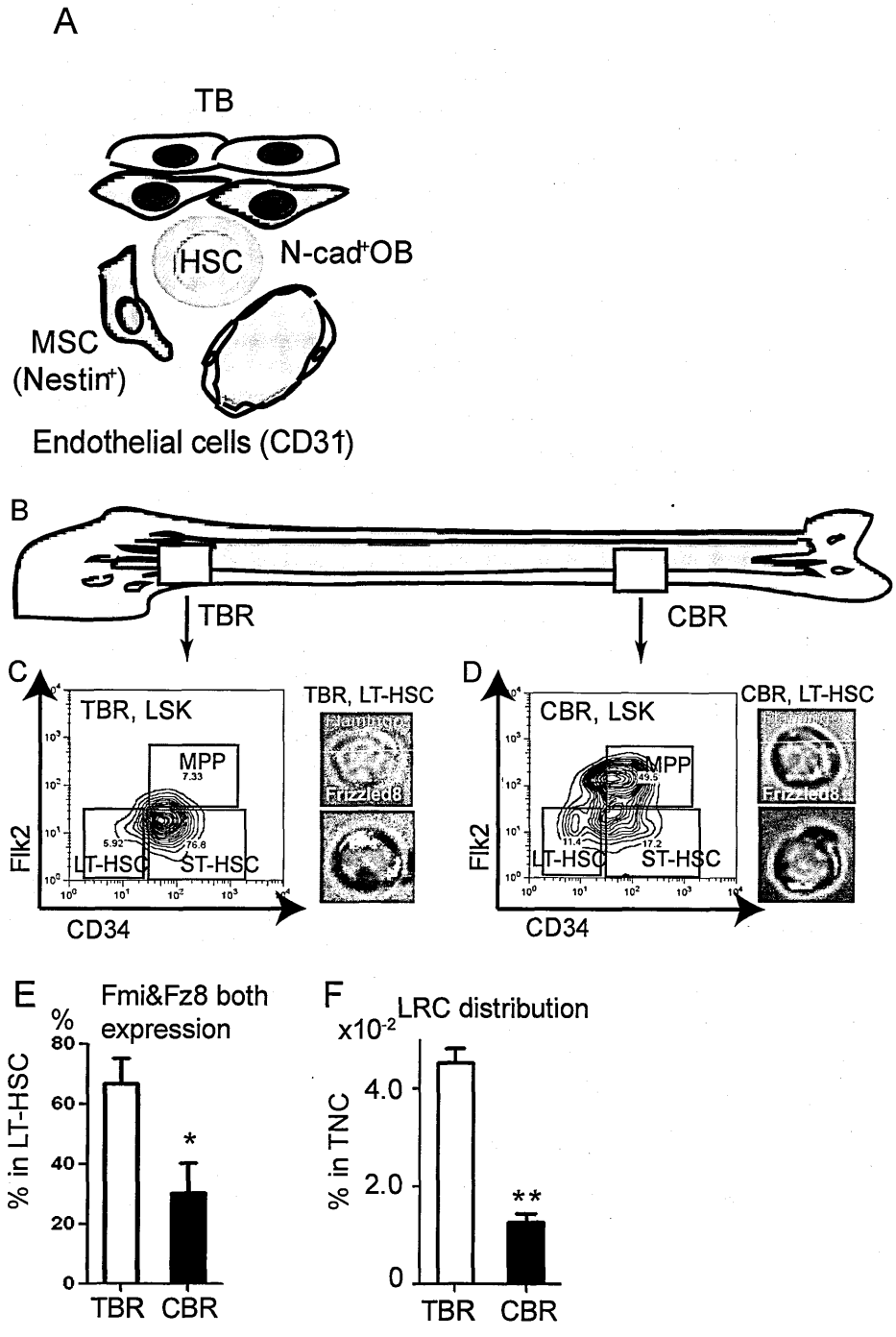

Figure 4-1 Distribution of Fmi and Fz8 expressing HSCs in bone

(A) HSC and niche components N-cad^{+}OBs, Nestin^{+}-MSCs, and endothelial cells (CD31^{+}). (B) Femur sagittal section showing TBR and CBR. (C-D) Sorted LT-HSCs from TBR (C) and CBR (D) and corresponding immunostaining of Fmi and Fz8. (E-F) Percentage of LT-HSCs (E) and H2B-GFP^{hi} LRCs in total nuclear cell (TNC) (F) expressing both Fmi and Fz8 from TBR and CBR. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

I next determined the distribution of quiescent LT-HSCs in the sagittal section of the femur. I identified quiescent LT-HSCs using the Scl-tTA-induced H2B-GFP^{hi} LRCs (H2B-GFP^{hi}LRCs) (Blanpain et al., 2004; Foudi et al., 2008; Tumbar et al., 2004; Wilson et al., 2008), and I compared the distribution of quiescent LT-HSCs between the TBR and the CBR (Figure 4-1F, 4-2A-B). Flow cytometry analysis showed the frequency of quiescent HSCs (H2B-GFP^{hi} LRCs) was more than 3-fold higher in the TBR than in the CBR (Figure 4-1F). Immunostaining confirmed this observation, revealing H2B-GFP^{hi} LRCs mainly in the TBR but not in the CBR endosteum (Figure 4-2A-B).

I then examined the correlation between the distribution of quiescent LT-HSCs (H2B-GFP^{hi}LRCs) and the distribution of niche components known to regulate HSCs, including endothelial cells, Nestin^{+} MSCs, and N-cad^{+}OBs (Kiel et al., 2005; Mendez-Ferrer et al., 2010; Wilson et al., 2008; Xie et al., 2009; Zhang et al., 2003a) (Figure 4-1A, 4-2D-P). Using a CD31-GFP endothelial reporter (established in our lab) and Nestin-GFP reporter (Mignone et al., 2004), I found that CD31-GFP^{+} endothelial cells and Nestin-GFP^{+} cells were distributed in both TBR and CBR without bias (Figure 4-2D-E, 4-2G-H). In contrast, N-cad^{+}OBs were predominantly detected in the TBR but rarely in the CBR (Figure 4-2J, L). The anti-N-cad monoclonal antibody (AbD13077) was newly generated by Suda's lab, and its specificity was confirmed by loss of N-cad staining in the N-cad knockout mice (Figure 4-2K, R). I detected expression of Fmi and Fz8 in N-cad^{+}OBs mainly in the TBR endosteum (yellow arrow, compare Figure 4-2J, N to 4-2L, O).

Next I compared the relationship between Fmi and other niche markers (Figure 4-2C, F, I, M, P, Q). I found that Fmi expression as shown by

immunostaining was almost absent in CD31-GFP^{+} endothelial cells, low in Nestin-GFP^{+} cells (Figure 4-2F, I, Q), and high in N-cad^{+}OBs (Figure 4-2M, Q). Fz8 was also expressed in N-cad^{+}OBs (Figure 4-2O-P). Notably, I detected Fmi and Fz8 at the interface between H2B-GFP^{hi}LRCs and N-cad^{+}OBs in the TBR endosteum (Figure 4-2C, P), supporting a role of Fmi and Fz8 in mediating a niche signal to regulate quiescent LT-HSCs.

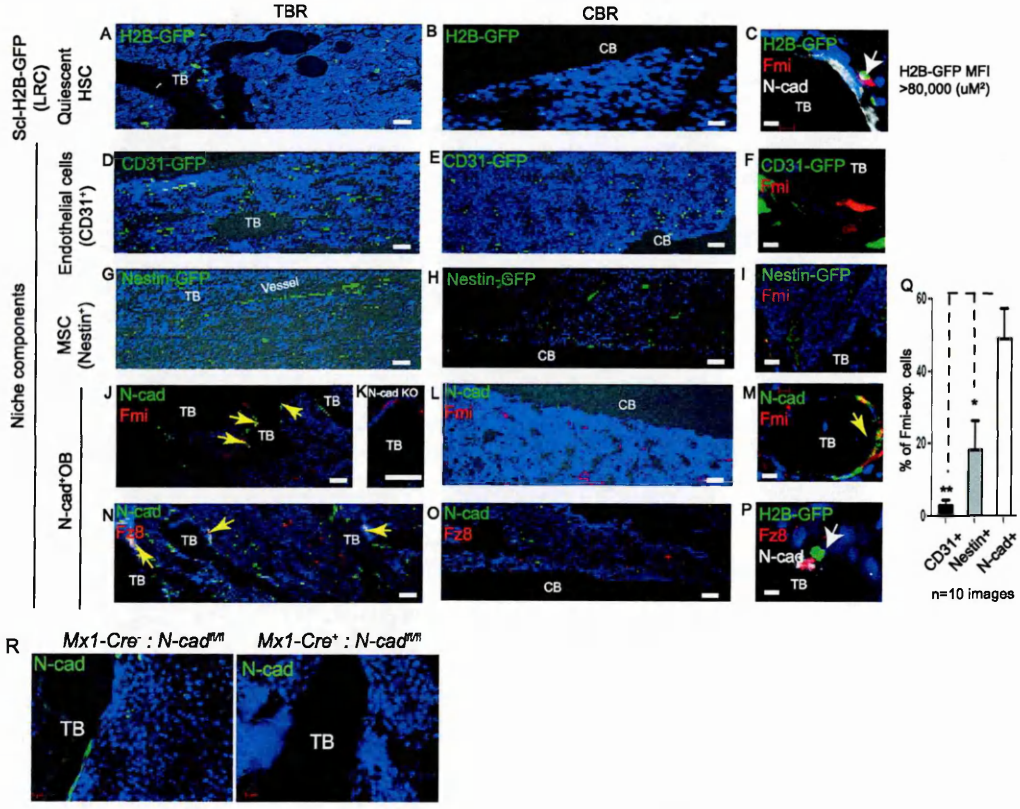

Figure 4-2 Distribution of quiescent HSCs and niche components

(A-P) Immunostaining of TBR and CBR with different HSC or niche component markers. (A-B) H2B-GFP^{hi} LRCs representing quiescent LT-HSCs. H2B-GFP signal was measured using mean fluorescence intensity (MFI) and MFI of H2B-GFP^{hi} LRCs was found to be >80,000 (uM^2). (D-E) CD31-GFP as endothelial reporter. (G-H) Nestin-GFP as MSC reporter. (J-L) N-cad staining, Fmi staining. (N-O) N-cad staining, Fz8 staining. (K) N-cad conditional knockout. Scale bar is 20uM. For enlarged images (C, F, I, M, P), scale bar is 5uM. (Q) Percentage of niche components expressing Fmi. Quantified from microscopy images. n=10 images. (R) N-cad staining for *Mx1-Cre*$^{-}$*:N-cad*$^{fx/fx}$ and *Mx1-Cre*$^{+}$*:N-cad*$^{fx/fx}$. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

The co-localization of Fmi and Fz8 was observed in H2B-GFP^{hi} LRCs attached to the bone surface of TBR (Figure 4-3A), which was further confirmed by consecutive sections revealing this co-localization at the interface between H2B-GFP^{hi} LRCs and N-cad^{+}OBs (Figure 4-3B). I took a 4-color 3D high-resolution image (Figure 4-3C) to confirm the *in vivo* co-localization of Fmi (red) and Fz8 (green) at the interface between H2B-GFP^{hi} LRCs (white nucleus) and N-cad^{+}OBs (blue). In addition, I used OP9 assay and observed the co-localization of Fmi and Fz8 at the interface between sorted H2B-GFP^{hi} Flk2^{-}LSKs and OP9 cells (Figure 4-3D) as well as sorted LT-HSCs and OP9 cells (Figure 4-3E). This co-localization was observed neither in H2B-GFP^{-}Flk2^{-}LSKs (Figure 4-3D) nor in ST-HSCs (Figure 4-3E). Presumably, this was due to lower expression levels of Fmi and Fz8 in these cells. In summary, quiescent LT-HSCs are found more in the TBR than in the CBR. Fmi and Fz8 are co-localized at the interface between quiescent LT-HSCs and N-cad^{+}OBs.

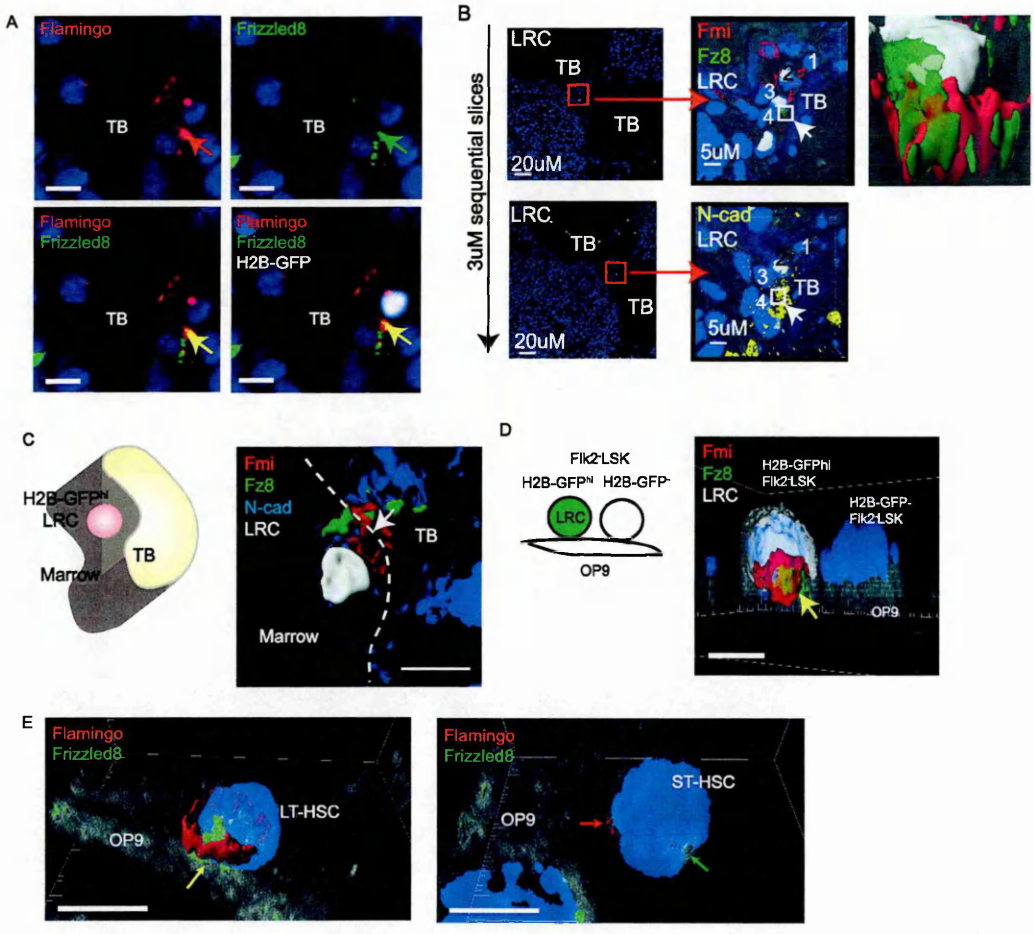

Figure 4-3 Co-localization of Fmi and Fz8 in quiescent HSCs

(A) Co-localization of Fmi and Fz8 at the interface between H2B-GFP^{hi} LRC and endosteum of TBR. (B) Co-localization of Fmi and Fz8 at the interface between H2B-GFP LRCs and N-cad^{+}OBs in sub-sequential sections. (C) High-resolution 3D images of H2B-GFP^{hi} LRC (white, MFI=122,009 uM^{2}), Fmi (red), Fz8 (green) and N-cad (blue) in TBR. Co-localization of Fmi and Fz8 at the interface between H2B-GFP^{hi} LRCs and N-cad^{+}OBs (white arrow). (D) High-resolution 3D image of sorted Flk2^{-}H2B-GFP^{hi}LSK and Flk2^{-}H2B-GFP^{-}LSK on OP9 cells. Co-localization of Fmi and Fz8 at the interface between Flk2^{-}H2B-GFP^{hi}LSK and OP9 cells (yellow arrow). (E) Co-localization of Fmi and Fz8 at the interface between LT-HSC and OP9 cells, but not between ST-HSC and OP9 cells. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

Chapter 5. N-cad$^+$OBs and HSCs maintain non-canonical Wnt signaling during homeostasis, but are attenuated in response to stress

In this chapter, I will analyze which Wnt ligands are produced from each niche component using RNA-sequencing technology. Also, I will analyze the dynamic change of Wnt ligand expression patterns in the niche following stress.

Fmi and Fz8 have been shown to mediate non-canonical Wnt signaling, which in turn suppresses canonical Wnt signaling (Morgan et al., 2003). Aparna Venkatraman used RNA-sequencing analysis to examine the expression patterns of ligands and inhibitors for canonical and non-canonical Wnt signaling in the niche components, including CD31$^+$VEGEFR2$^+$ cells (endothelial progenitor cells), Nestin-GFP$^+$ MSC-like cells, N-cad$^+$ OBs, and Col2.3-GFP$^+$ mature OBs (Kalajzic et al., 2002; Lyden et al., 2001; Mendez-Ferrer et al., 2010; Xie et al., 2009; Zhang et al., 2003a) (Figure 5-1A). Expression level was measured by Fragments per Kilobase of exon per Million fragments mapped (FPKM) (Trapnell et al., 2010). Expression of canonical Wnt ligands overall was either absent or low (3-6 FPKM) in all three niche components (Figure 5-1B, Table 5-1). There were expressions of *Wnt2b* and *Wnt7b* only in N-cad$^+$OBs; *Wnt9a* in both N-cad$^+$OBs and mature OBs; and *Wnt10b* in Nestin-GFP$^+$, N-cad$^+$OBs, and mature OBs (Figure 5-1B). In contrast, expression of canonical Wnt inhibitors was much higher (15-168 FPKM) and mainly in Nestin-GFP$^+$ cells, N-cad$^+$OBs, and mature OBs, with N-cad$^+$OBs expressing the highest levels. For example, expressions of *Sfrp4* and *Dkk3* were 6 and 5 times higher respectively in N-cad$^+$OBs than in Nestin-GFP$^+$ cells. *Wif1* expression in N-cad$^+$OBs

was 6.5-fold and 1.6-fold higher than in CD31$^+$VEGEFR2$^+$ and Nestin-GFP$^+$ cells respectively (Figure 5-1C). *Wif1* as a non-canonical Wnt inhibitor was 2- and 5-fold higher than *Dkk3* and *Sfrp4* respectively in Nestin$^+$ cells, suggesting a favored canonical Wnt environment maintained by Nestin$^+$ cells (Hsieh et al., 1999). In contrast, *Sfrp4*, a canonical Wnt inhibitor, was 3-fold higher than *Wif1* in N-cad$^+$OBs (Figure 5-1C), suggesting a dominant non-canonical Wnt signaling in N-cad$^+$OBs.

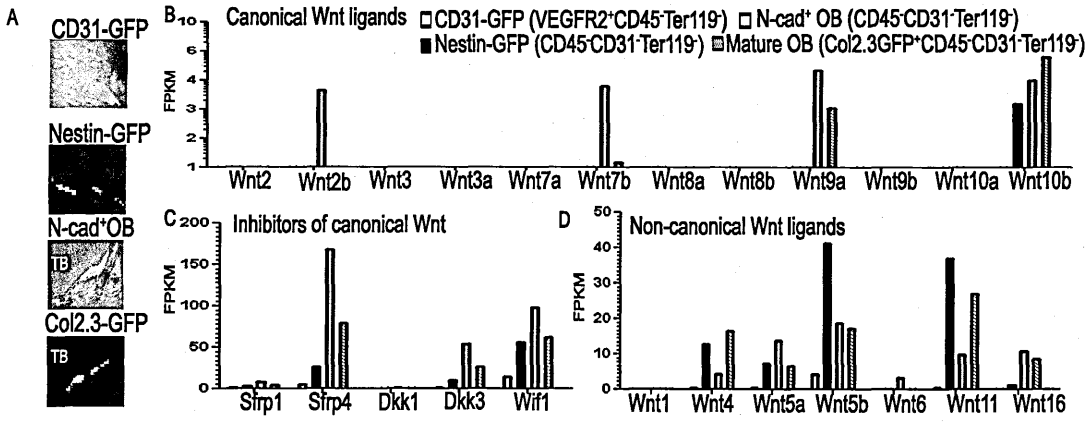

Figure 5-1 RNA-sequencing of niche components for Wnt genes

(A) BM section with CD31-GFP (green), Nestin-GFP (green), N-cad$^+$OBs (green), and Col2.3-GFP (green) respectively. We used FACS to sort these cells [CD31-GFP$^+$ (VEGFR2$^+$CD45$^-$Ter119$^-$) cells, Nestin-GFP$^+$ (CD45$^-$CD31$^-$Ter119$^-$) cells, N-cad$^+$OBs (CD45$^-$CD31$^-$Ter119$^-$) and mature OBs Col2.3-GFP$^+$ (CD45$^-$CD31$^-$Ter119$^-$).] and analyzed with RNA-sequencing. (B-D) RNA-sequencing analysis of niche components for canonical Wnts (B), canonical Wnt inhibitors (C), and non-canonical Wnts (D). Expression level was shown by FPKM (Fragments per Kilobase of exon per Million fragments mapped). CD45: hematopoietic cell marker, CD31: endothelial cell marker, Ter119: red blood cell marker (Modified from Sugimura et al., Cell 150, 351-365, 2012)

Table 5-1 RNA-sequencing FPKM value of niche components

Canonical Wnt ligands in niche components

	CD31-GFP (VEGFR2+)	Nestin-GFP (CD31-)	N-cad+OB	Total OB
Wnt2	0.09	0.11	0	0.13
Wnt2b	0.41	0.4	3.39	0.84
Wnt3	0	0	0	0
Wnt3a	0	0	0	0
Wnt7a	0	0	0	0
Wnt7b	0	0.76	3.62	1.08
Wnt8a	0	0.03	0	0.14
Wnt8b	0	0.03	0	0
Wnt9a	0.82	0.98	4.68	2.59
Wnt9b	0.34	0	0	0
Wnt10a	0	0.11	0.28	0
Wnt10b	0.24	2.77	4	5.79

Non-canonical Wnt ligands in niche components

	CD31-GFP (VEGFR2+)	Nestin-GFP (CD31-)	N-cad+OB	Total OB
Wnt1	0.03	0.03	0.09	0.05
Wnt4	0.59	12.7	4.29	16.6
Wnt5a	0.43	7.4	13.8	6.6
Wnt5b	4.31	41.4	18.8	17.4
Wnt6	0	0.09	3.22	0.13
Wnt11	0.53	37	9.76	27.1
Wnt16	0.07	1.17	10.7	8.64

Canonical Wnt inhibitors in niche components

	CD31-GFP (VEGFR2+)	Nestin-GFP (CD31-)	N-cad+OB	Total OB
Sfrp1	1.02	2.73	8.58	4.07
Sfrp4	5.27	26.3	168	79.1
Dkk1	0.05	0.51	0.92	0.51
Dkk3	1.25	10	54.6	25.7
Wif1	14.6	56.4	98	62.6

In summary, canonical Wnt ligands were generally expressed at low levels, and their activity was most likely suppressed by very high levels of inhibitors (Figure 5-1B-C). Non-canonical Wnt ligands overall were expressed at comparable levels in Nestin-GFP$^+$ cells, mature OBs, and N-cad$^+$OBs, except for *Wnt6* and *Wnt16* which were predominantly expressed in N-cad$^+$OBs (Figure 5-1D). CD31$^+$VEGEFR2$^+$

endothelial cells expressed low level *Wnt5b* (Figure 5-1D). These observations suggest that the N-cad^{+}OB niche provides a microenvironment in which canonical Wnt signaling is suppressed and non-canonical Wnt signaling is predominant in homeostasis.

N-cad^{+}OBs in the TBR have been implicated in maintaining quiescent HSCs as evidenced by their physical location adjacent to BrdU^{+} LRCs, H2B-GFP^{hi} LRCs, and transplanted HSCs (Arai et al., 2004; Wilson et al., 2008; Xie et al., 2009; Zhang et al., 2003a). During stress, however, quiescent HSCs can be activated and undergo expansion. For example, expansion of N-cad^{+}OBs precedes the activation and subsequent expansion of HSCs (Dominici et al., 2009) (Figure 5-2A-B). I therefore compared the change in expression levels of canonical and non-canonical Wnt ligands and inhibitors in N-cad^{+}OBs under 5FU-induced BM (BM) stress. Four days post 5FU treatment, I found that N-cad^{+}OBs were resistant to 5FU treatment and subsequently expanded (more than 5-fold increase in both frequency and number in bone) (Figure 5-2C-E). In contrast, N-cad^{-}OBs were sensitive to 5FU treatment and significantly declined ~7-fold post treatment (Figure 5-2C). This result suggests that N-cad^{+}OBs are normally in a quiescent state and thereby resistant to 5FU treatment.

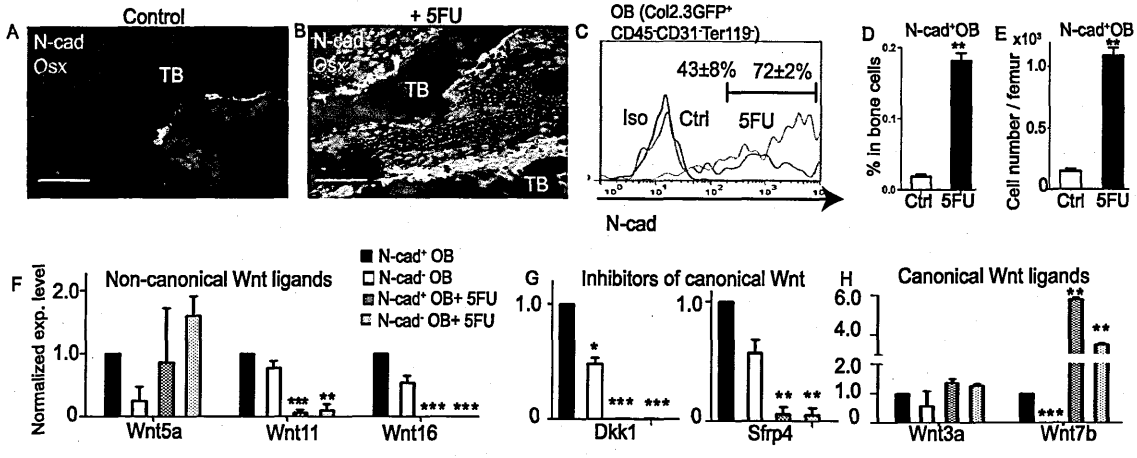

Figure 5-2 Changes in N-cad$^+$OBs post 5FU

(A-B) Immunostaining of TBR with N-cad (green), OB marker Osterix (Osx) (red) and DAPI (blue) in control (A) and 4 days post 5FU treatment (B). Scale bar is 20uM. (C) Flow cytometric analysis of the change in percentage of N-cad$^+$ OBs in total OBs without or with 5FU treatment. The cells were gated from Col2.3GFP$^+$CD45$^-$CD31$^-$Ter119$^-$ OBs. (D-E) Frequency (D) and number (E) of N-cad$^+$OBs in bone cells post 5FU. (F-H) qRT-PCR for non-canonical Wnts (F), inhibitors of canonical Wnt (G) and canonical Wnts (H) among N-cad$^+$OBs and N-cad$^-$OBs with or without 5FU. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

The correlation between an increase in N-cad$^+$OBs (Figure 5-2C-E) and activation and expansion of HSCs (Dominici et al., 2009) post 5FU treatment suggested that 5FU could also induce dynamic changes in the expression of Wnt ligands in N-cad$^+$OBs. To test this idea, I sorted N-cad$^+$OBs and N-cad$^-$OBs with or without 5FU treatment and examined the expression of Wnt ligands and inhibitors using qRT-PCR (Figure 5-2F-H). I found that non-canonical ligands *Wnt11* and *Wnt16,* as well as canonical Wnt inhibitors *Dkk1* and *Sfrp4,* were diminished (Figure 5-2F-G, 5-3). However, canonical *Wnt7b* increased 6-fold post 5FU treatment (Figure 5-2H). These observations show that 5FU treatment leads to a decline of non-canonical Wnt signals but an increase in canonical Wnt signaling via upregulation of canonical Wnts and downregulation of inhibitors.

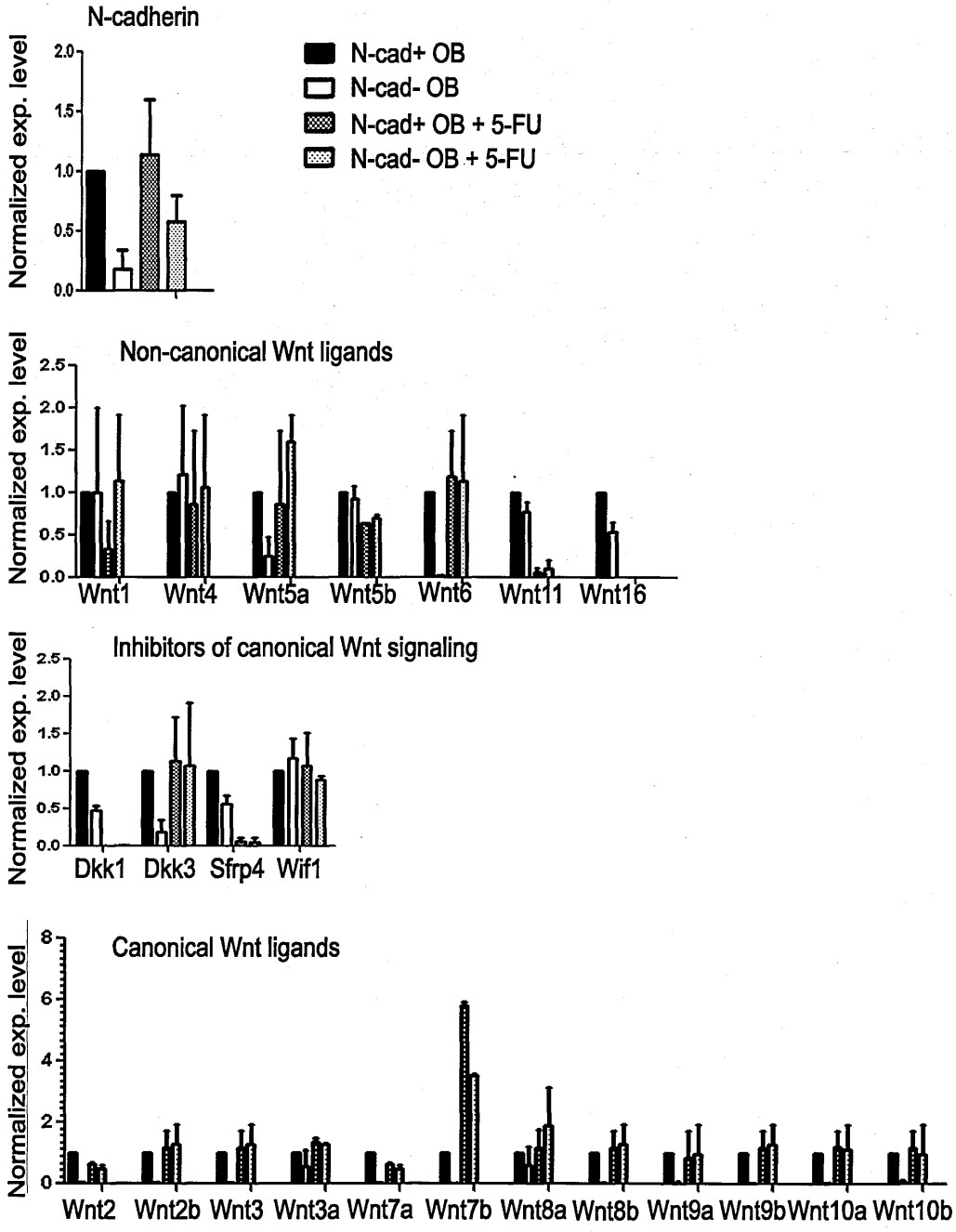

Figure 5-3 Full gene list for Figure 5-2

qRT-PCR of N-cad, non-canonical Wnt ligands, canonical Wnt inhibitors, and canonical Wnt ligands in N-cad^{+}OBs, N-cad^{-}OBs with or without 5FU. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

Next, I investigated the impact of 5FU on Fmi and Fz8 in HSCs. I isolated LT-HSCs (CD34^{-}Flk2^{-}LSK) from TBR and CBR (Figure 5-4A) and used

immunostaining to compare the co-localization of Fmi and Fz8 before and after 5FU treatment (Figure 5-4C). Observing Fmi and Fz8 co-localization post 5FU by high-resolution 3D image (Figure 5-4B), I found an ~8-fold decline in Fmi-Fz8 co-localization in LT-HSCs from TBR and a 2-fold decline of that in CBR (Figure 5-4D). This observation was explained by the reduction of both Fmi and Fz8 mRNA in LT-HSCs post 5FU. In addition, Fz8 protein (Figure 5-4G-H) and mRNA (Figure 5-4I) were reduced by 50%, and Fmi mRNA was increased by 20% in N-cad^{+}OBs post 5FU (Figure 5-4I). The data indicate that non-canonical Wnt receptors Fmi and Fz8 are reduced in LT-HSCs post 5FU treatment.

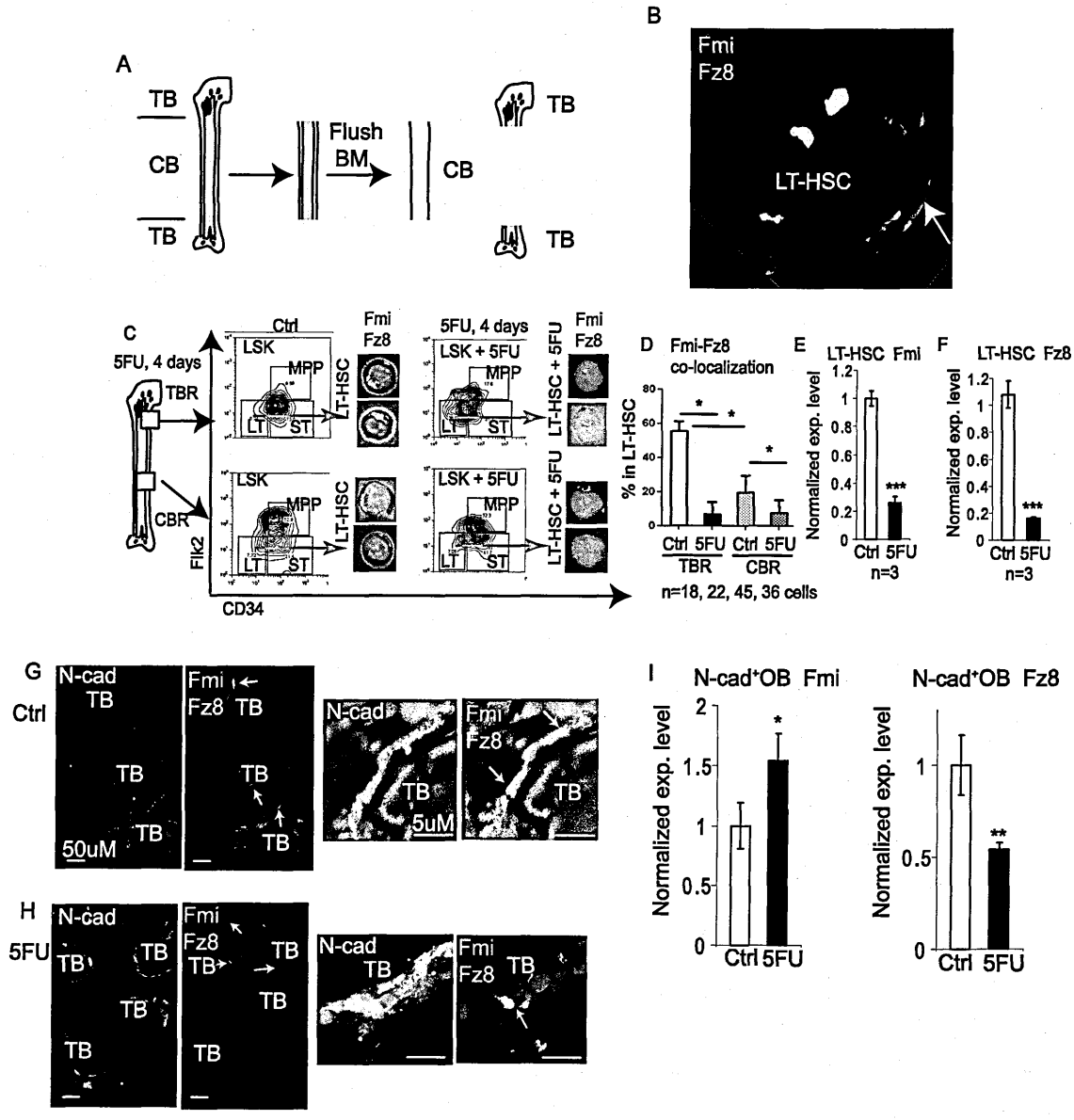

Figure 5-4 5FU-induced change of Fmi and Fz8 expression in HSC and niche

(A) Isolation of LT-HSCs from TBR and CBR. TBR was removed first, then BM was flushed. (B) 3D confocal image of Fmi and Fz8 co-localization in LT-HSCs. (C) Representative flow panels and image of Fmi and Fz8 co-staining of LT-HSCs from TBR (upper left), central marrow of CBR (lower left), TBR 4 days post 5FU treatment (upper right), and central marrow of CBR 4 days post 5FU (lower right); Fmi (red), Fz8 (green), and DAPI (blue). (D) Percentage of Fmi and Fz8 co-localization in LT-HSCs from TBR (white), central marrow of CBR (black), TBR 4 days post 5FU treatment (smaller dots), and central marrow of CBR 4 days post 5FU (larger dots). n=18, 22, 45, 36 cells. (E-F) Fmi expression level (E) and Fz8 expression level (F) in LT-HSCs 4 days post 5FU. (G-H). Fmi and Fz8 immunostaining of TBR in control (G) and 4 days post 5FU (H). (I) qRT-PCR for Fmi and Fz8 in N-cad^{+}OB post 5FU. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

Next, I asked whether 5FU induced a reduction of non-canonical Wnt signaling and subsequent upregulation of canonical Wnt signaling in LT-HSCs (Figure 5-5A). As the Ca^{2+}-NFAT pathway is one of the major molecular downstream events of non-canonical Wnt signaling in adult tissue (Dejmek et al., 2006; Gregory et al., 2010), I measured intracellular Ca^{2+} level by Fluo-3 (Minta et al., 1989). LT-HSCs post 5FU treatment showed a 2.5-fold increase of intracellular Ca^{2+} level (Figure 5-5B) and an 8-fold increase of NFATc1 nuclear translocation (Figure 5-5C). The downstream target genes of NFAT, *IFNγ* and *Cox2*, were upregulated 6- and 2-fold respectively in LT-HSCs post 5FU treatment (Figure 5-5D). I also examined *IFNγ* expression in cytotoxic T cells and regulatory T cells (Treg cells) that were recently reported to be a niche component (Treg, $CD4^+CD25^+$) (Fujisaki et al., 2011) (Figure 5-5E). Notably, *IFNγ* expression was upregulated in Treg cells but not in cytotoxic T cells (Figure 5-5E), indicating sources of IFNγ include both autocrine from HSCs as well as paracrine from surrounding Treg cells.

To measure canonical Wnt signaling in LT-HSCs post 5FU treatment, I immunostained the sorted LT-HSCs with β-catenin-pS552, which was phosphorylated by phosphor-Akt (pAKT) and became an active form in nucleus (He et al., 2007). pAkt staining was observed in more than 70% of LT-HSCs post 5FU treatment but in only 10% of control LT-HSCs (Figure 5-5F). β-catenin-pS552 was detected in more than 50% of LT-HSCs post 5FU treatment and in 20% of control LT-HSCs (Figure 5-5G). This observation was confirmed by TOP-Gal staining which showed more than a 2.5-fold increase of signal intensity in LT-HSCs post 5FU treatment (Figure 5-5H). Furthermore, canonical Wnt target Axin2 (Luis et al., 2011) increased 8-fold in LT-HSCs post 5FU treatment (Figure 5-5I). These observations

indicate that 5FU induces a decline of non-canonical Wnt signaling (Figure 5-5B-D) and an increase of canonical Wnt signaling (Figure 5-5F-I) in LT-HSCs.

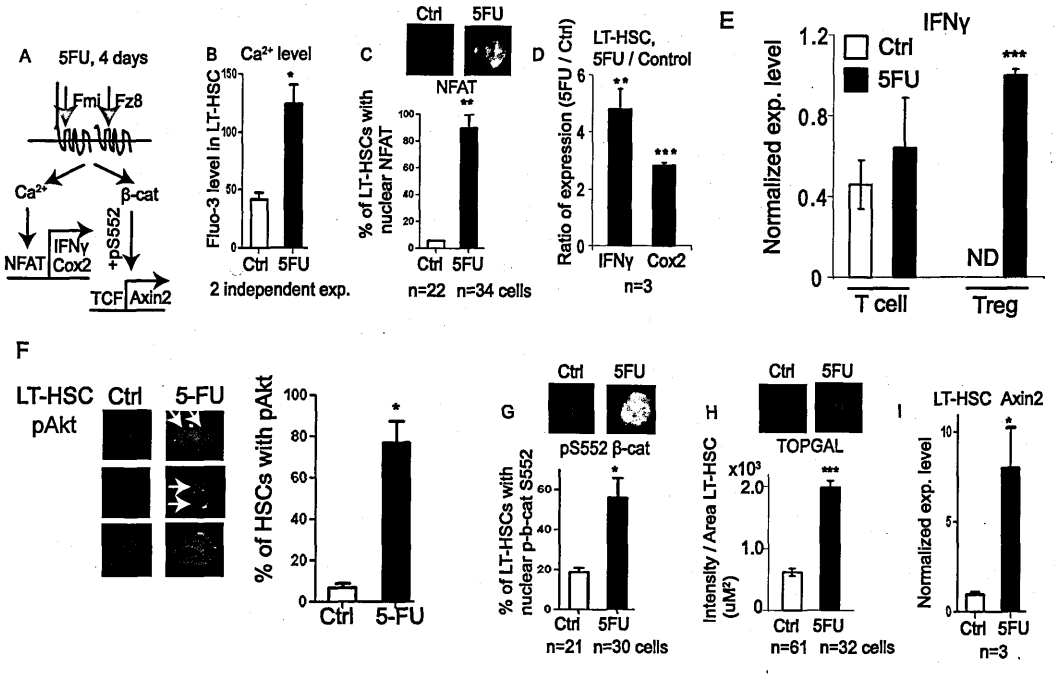

Figure 5-5 5FU-induced change of canonical and non-canonical Wnt signaling in HSC

(A) Non-canonical Wnt signaling (Ca^{2+}-NFAT pathway) and canonical Wnt signaling (β-catenin-TCF-Axin2). Non-canonical Wnt receptors Fmi and Fz8 were declined post 5FU. (B) Intracellular Ca^{2+} level in LT-HSCs post 5FU. 2 independent experiments. Comparison of protein and mRNA levels between control and 5FU treated samples (C) NFAT staining of LT-HSCs (D) qRT-PCR analysis of NFAT targets, *IFNγ* and *Cox2* in LT-HSCs. Reactions were triplicated. (E) qRT-PCR for *IFNγ* in T cells and Treg ($CD4^+CD25^+$). (F) Immunostaining of pAkt in LT-HSCs post 5FU. White arrow indicates dividing LT-HSCs post 5FU. (G) β-catenin-pS552 staining of LT-HSCs. (H) TOP-GAL staining of LT-HSCs. (I) qRT-PCR analysis of *Axin2* in LT-HSCs. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

Chapter 6. Flamingo maintains quiescent long-term HSCs *in vivo*

In this chapter, I will analyze the functional role of *Fmi* in HSC maintenance *in vivo*. I used the mouse model of *Fmi* gene conventional knockout.

Since Fmi-Fz8 co-localization is enriched in quiescent HSCs and in the niche that expresses non-canonical Wnt ligands, I hypothesized that Fmi may have a role in maintaining HSC quiescence. My preliminary results of knockdown of Fmi led to a reduction in LT-HSCs but an increase in ST-HSCs and MPPs, and functionally resulted in more than a 50-60% decrease of engraftment in repopulation assay (Figure 6-1A). To confirm this, I used *Fmi/Celsr2* conventional knockout mouse model. The gender and age (>40 days old) of mice were matched for the analysis (Figure 6-1B). The frequency of LT-HSCs decreased 60% (Figure 6-1B). In addition, the number of LT-HSCs decreased 80%, and the numbers of ST-HSCs and MPPs declined as well in the $Fmi^{-/-}$ mice (Figure 6-1C). The results from both knockdown and knockout suggest loss of LT-HSC function followed by consequent loss of ST-HSCs and MPPs, which correlates with predominant expression of *Fmi* in quiescent LT-HSCs. I used a hypoxic-related HSC marker GRP78 to stain LT-HSCs and observed a decline in $GRP78^{+}$ population from 31.3 ± 0.3% (control) to 11.8 ± 1.1% ($Fmi^{-/-}$) (Figure 6-1D-E). In line with this finding, I observed an increase of mitochondrial activity in $Fmi^{-/-}$ LT-HSCs (Figure 6-1F). All these suggested an increase in HSC activity when Fmi was lost. I therefore performed a cell cycle analysis and found that within $Flk2^{-}$LSK HSCs, the percentage of quiescent (G0) cells declined from 23 ± 0.71% (control) to 15 ± 1.1% ($Fmi^{-/-}$) (Figure 6-1G-H).

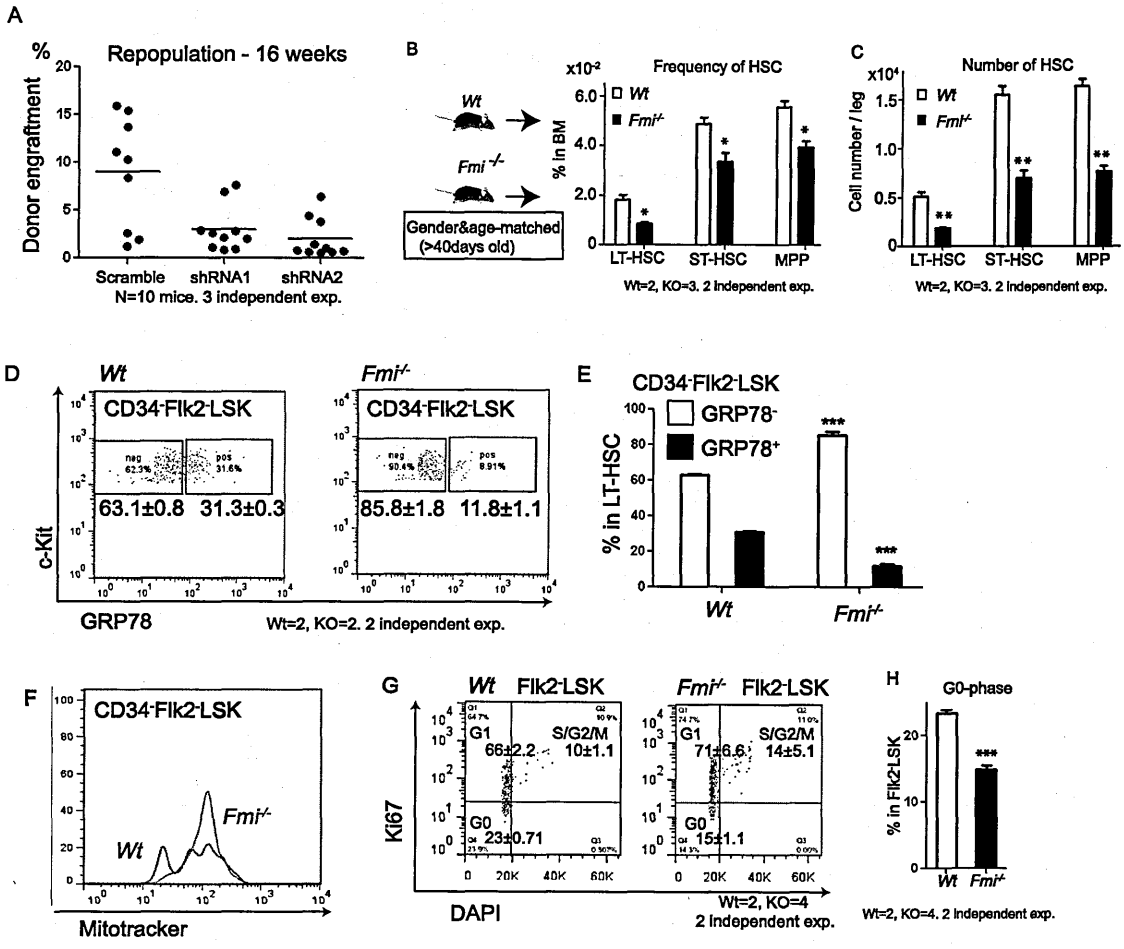

Figure 6-1 Analysis of primary *Fmi*$^{-/-}$ mice

(A) Transplantation of Fmi knockdown over 16 weeks. N=10 mice. 3 independent experiments. (B-C) Analyses of conventional Fmi knockout mice. Frequency (B) and number (C) of HSCs in TBR. (D) GRP78 analysis of LT-HSCs comparing *Fmi*$^{-/-}$ mice and *Wt*. Wt=2, KO=2 mice, 2 independent experiments. (E) Percentage of GRP78^{+} and GRP78^{-} LT-HSCs. (F) Mitochondrial activity assay comparing *Fmi*$^{-/-}$ mice and *Wt*. (G) Cell-cycle analysis of Flk2^{-}LSK comparing *Fmi*$^{-/-}$ mice and *Wt*. Wt=2, KO=4 mice. 2 independent experiments. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

Next, I transplanted 100 LSK (alive) enriched with HSPCs (CD45.2) with rescue $2x10^5$ BM (CD45.1) into lethally irradiated recipient mice (CD45.1) (Figure 6-2A). Homing efficiency analysis of the recipient mice 16 hours posttransplantation confirmed that knockout of Fmi did not affect HSPC homing to BM and spleen (Figure 6-2B). After 16 weeks, Fmi knockout reduced 80% of the hematopoietic reconstitution from donor-derived HSCs (Figure 6-2A). The analyses of recipients

showed an 80% decrease of donor-derived LT-HSCs and a 70% decrease of donor-derived ST-HSCs in frequency and number (Figure 6-2C-D). In addition, the loss of HSCs was not due to apoptosis, since knockout did not increase AnnexinV^{+}SytoxGreen^{+} LT-HSCs (Figure 6-2E).

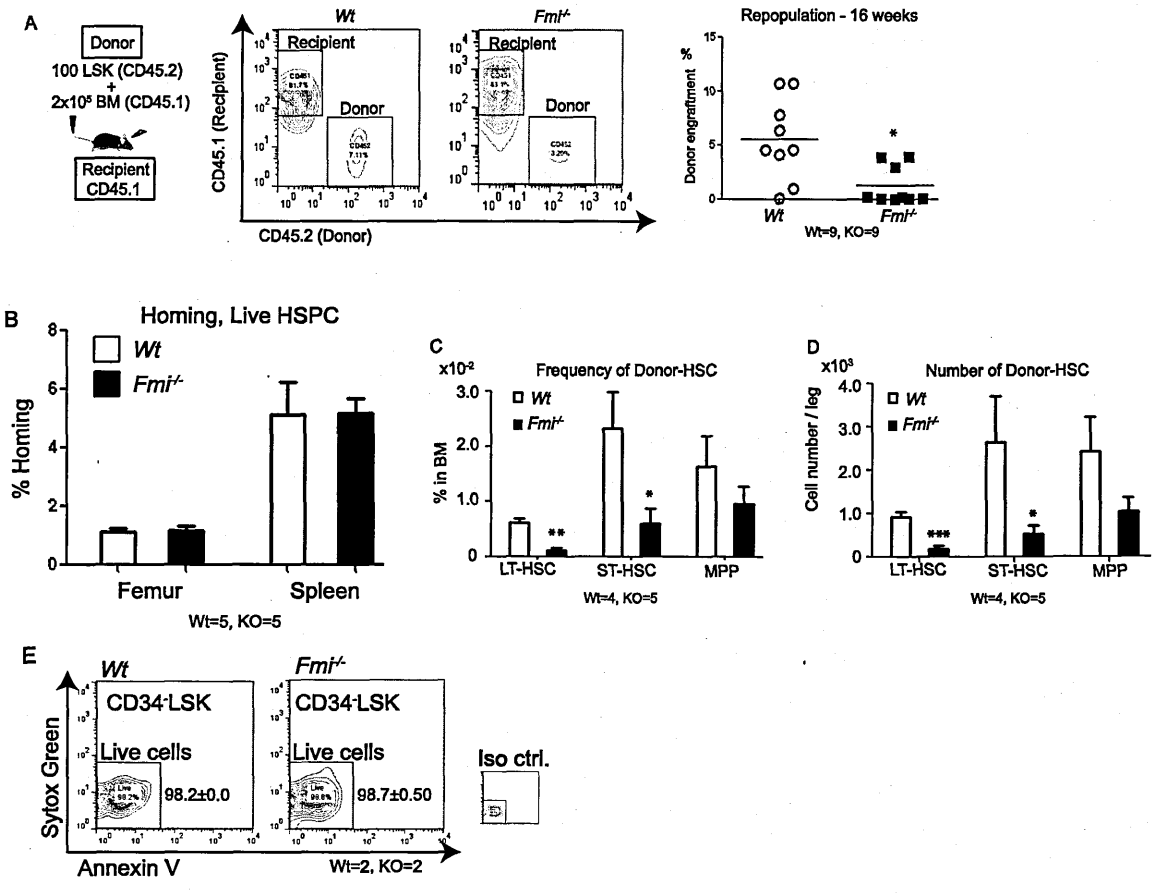

Figure 6-2 Transplantation analysis of *Fmi*$^{-/-}$ mice

(A) Chimerism analysis of transplantation: 100 LSK (CD45.2) + 2x10^{5} BM (CD45.1). Recipients were lethally irradiated (CD45.1). The repopulation was analyzed 16 weeks post injection. (B) Homing analysis of live CFDA^{+}HSPC (7AAD^{-}CFDA^{+}LSK) comparing *Fmi*$^{-/-}$ mice and *Wt*. Wt=5, KO=5 mice. (C-D) Frequency (C) and number (D) of donor-HSCs 17 weeks post transplantation. (E) Apoptosis assay with SytoxGreen and AnnexinV staining of LT-HSCs comparing *Fmi*$^{-/-}$ mice and Wt. Wt=2, KO=2 mice. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

I next asked whether H2B-GFP^{hi} LRCs (quiescent HSCs) were affected by Fmi knockout. H2B-GFP^{hi} LRCs were predominantly observed in TBR in Wt, but less so in *Fmi*$^{-/-}$ (Figure 6-3A-B, D-E). The H2B-GFP signal intensity was

significantly reduced in *Fmi*$^{-/-}$ LT-HSCs, suggesting that label retention was lost due to decrease of quiescence in HSCs (Figure 6-3G). The frequency of H2B-GFP^{hi} LRCs in *Fmi*$^{-/-}$ was decreased 8-fold in TBR and 10-fold in CBR (Figure 6-3H). Within TBR in Wt control, 65% of H2B-GFP^{hi} LRCs were in direct contact with N-cad^{+}OBs, which was reduced 20% in *Fmi*$^{-/-}$ (Figure 6-3I). The localization of Fz8 protein was observed at the interface between H2B-GFP^{hi} LRCs and N-cad^{+}OBs in Wt (Figure 6-3C); however, its distribution became random in remaining *Fmi*$^{-/-}$ H2B-GFP^{hi} LRCs (Figure 6-3F). These observations provide further evidence to support the conclusion that *Fmi* facilitates the maintenance of quiescent LT-HSCs *in vivo*.

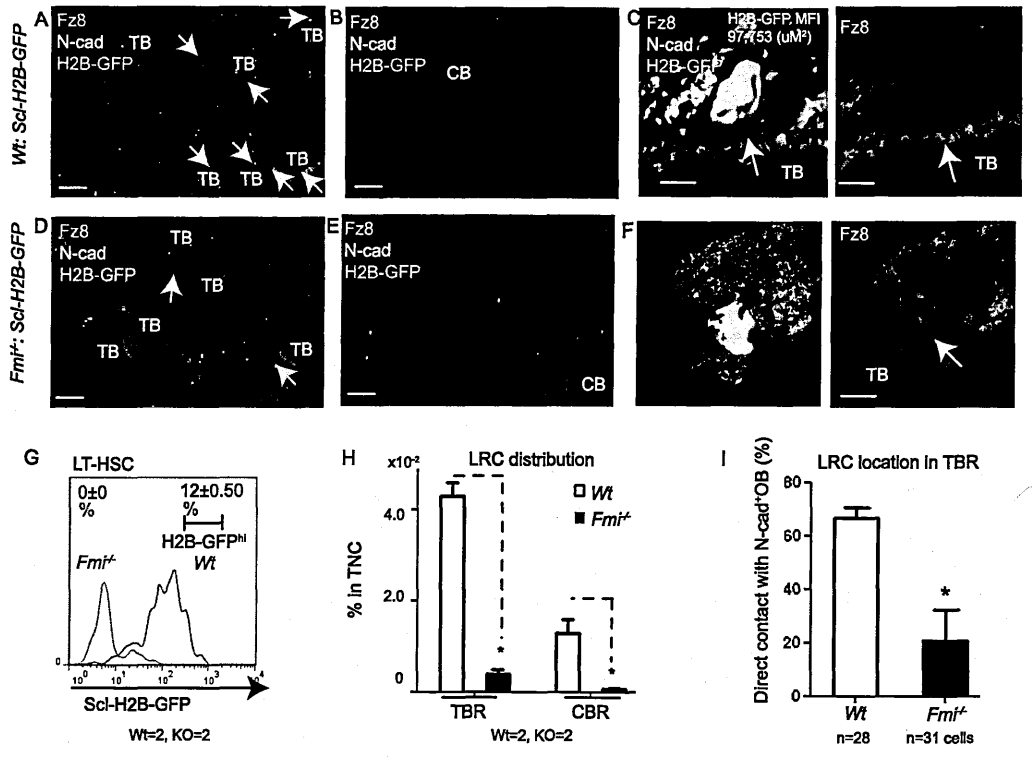

Figure 6-3 Label retention of *Fmi*$^{-/-}$ HSC

(A-F) Immunostaining of Fz8, N-cad, and H2B-GFP^{hi} LRC in TBR and CBR from *Scl-H2B-GFP* (A-C) and *Fmi*$^{-/-}$*: Scl-H2B-GFP* (D-F) mice. Scale bar is 20uM (A-B, D-E) or 5uM (C, F). High-resolution 3D image of localization of Fz8 (red arrow) in H2B-GFP^{hi} LRC (C, F). (G) Flow cytometric analysis of H2B-GFP signal in LT-HSCs from *Wt* and *Fmi*$^{-/-}$. (H) H2B-GFP^{hi} LRC distribution in TBR and CBR from *Wt* and *Fmi*$^{-/-}$. Wt=2, KO=2 mice. (I) Frequency of H2B-GFP^{hi} LRCs directly contacting with N-cad^{+}OBs in TBR from *Wt* or *Fmi*$^{-/-}$ mice. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

Chapter 7. Frizzled8 maintains quiescent long-term HSCs *in vivo*

In this chapter, I will analyze the functional role of *Fz8* in HSC maintenance *in vivo*, using the conventional *Fz8* knockout mouse model.

Since subcellular distribution of Fz8 is regulated by Fmi in quiescent LT-HSCs (H2B-GFP^{hi}Flk2^{-}LSK), I examined the functional role of Fz8 in HSCs using Fz8 conventional knockout mouse model. The gender and age (>40 days old) of mice were matched for the analyses (Figure 7-1A). The frequency of LT-HSCs decreased 40%. In addition, the number of LT-HSCs decreased 50%, and the numbers of ST-HSCs and MPPs declined as well in the *Fz8*$^{-/-}$ mice (Figure 7-1B). The results from *Fz8* knockout suggest loss of LT-HSCs followed by subsequent loss of ST-HSCs and MPPs, which correlates with predominant expression of *Fz8* in quiescent LT-HSCs. I performed a cell cycle analysis and found that within Flk2^{-}LSK HSCs, the percentage of quiescent (G0) cells declined from 22 ± 2.9% (control) to 16 ± 1.9% (*Fz8*$^{-/-}$) (Figure 7-1C-D).

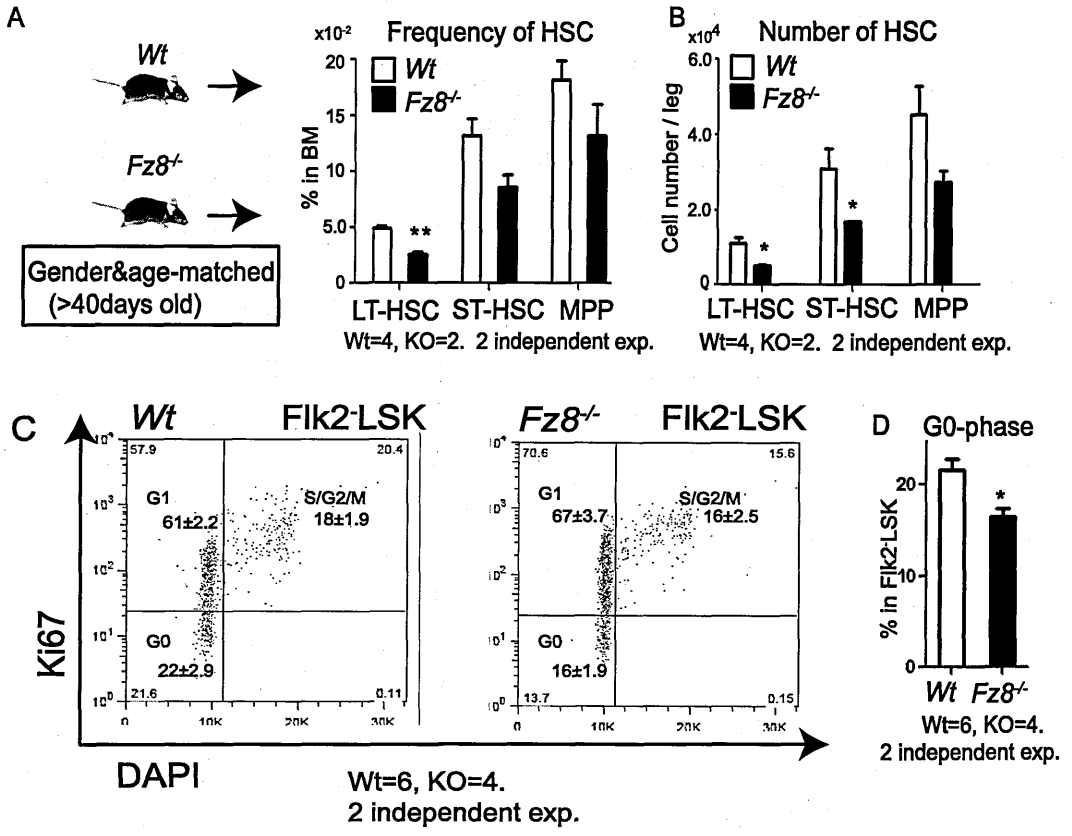

Figure 7-1 Analysis of primary *Fz8*$^{-/-}$ mice

(A-D) Analyses of conventional Fz8 knockout mice. Frequency (A) and number (B) of HSCs in BM. DNA content (DAPI) versus Ki67 staining of Flk2^{-}LSK cells to compare percentage of G0 phase cells (C-D). (Modified from Sugimura et al., Cell 150, 351-365, 2012)

Next, I transplanted 100 LSK (alive) enriched with HSPCs (CD45.2) with rescue 2x10^5 BM (CD45.1) into lethally irradiated recipient mice (CD45.1) (Figure 7-2A). Homing efficiency analysis of the recipient mice 16 hours posttransplantation confirmed that knockout of Fz8 did not affect HSPC homing to BM and spleen (Figure 7-2B). After 16 weeks, the *Fz8*$^{-/-}$ group reduced 70% of the hematopoietic reconstitution from donor-derived HSCs (Figure 7-2A). The analyses of recipients showed a 70% decrease of donor-derived LT-HSCs and a 70% decrease of donor-derived ST-HSCs in frequency and number (Figure 7-2C-D). In addition, the loss of

HSCs was not due to apoptosis, since *Fz8* knockout did not increase AnnexinV^{+}SytoxGreen^{+} LT-HSCs (Figure 7-2E).

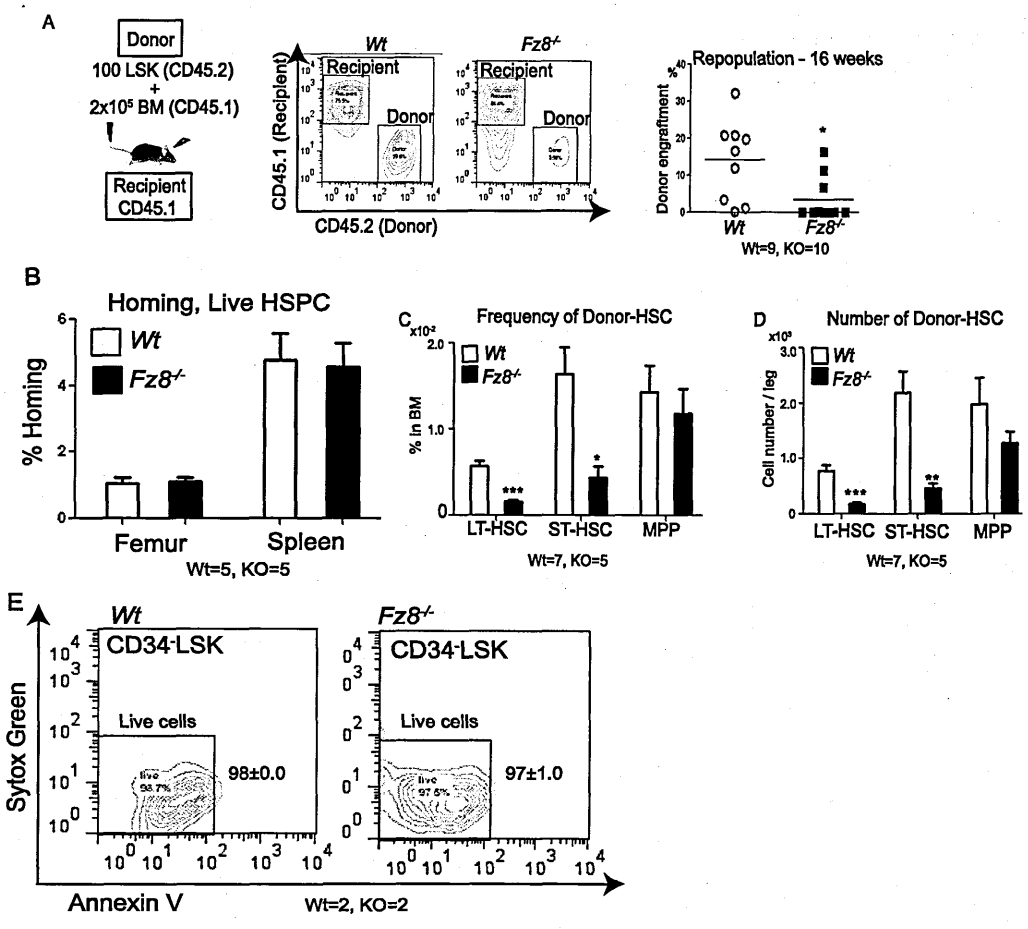

Figure 7-2 Transplantation analysis of *Fz8*$^{-/-}$ mice

(A) Chimerism analysis of transplantation: 100 LSK (CD45.2) + 2x10^{5} BM (CD45.1). Recipients were lethally irradiated (CD45.1). The repopulation was analyzed 16 weeks post injection. (B) Homing analysis of live CFDA^{+}HSPC (7AAD^{-}CFDA^{+}LSK) comparing *Fz8*$^{-/-}$ mice and *Wt*. Wt=5, KO=5 mice. (C-D) Analyses of recipient mice 20 weeks post transplantation. Frequency (C) and number (D) of donor-HSC. (E) Apoptosis assay with SytoxGreen and AnnexinV staining of LT-HSCs comparing *Fmi*$^{-/-}$ mice and *Wt*. Wt=2, KO=2 mice. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

I next asked whether H2B-GFP^{hi} LRCs (quiescent HSCs) were affected by Fz8 knockout. H2B-GFP^{hi} LRCs were predominantly observed in TBR in Wt, but less so in *Fz8*$^{-/-}$ (Figure 7-3A-B, D-E). The H2B-GFP signal intensity was significantly reduced in *Fz8*$^{-/-}$ LT-HSCs, suggesting that label retention was lost due to decrease in HSC quiescence (Figure 7-3G). The frequency of H2B-GFP^{hi} LRCs in

Fz8$^{-/-}$ was decreased 6-fold in TBR and 8-fold in CBR (Figure 7-3H). Within TBR in Wt, 63% of H2B-GFP^{hi} LRCs were in direct contact with N-cad^{+}OBs, which was reduced 10% in *Fz8*$^{-/-}$ mice (Figure 7-3I). The localization of Fmi protein was not affected in *Fz8*$^{-/-}$ LRCs (Figure 7-3C, F), further supporting that the function of Fmi is to determine Fz8 distribution, but Fmi distribution is not affected by Fz8. Taken together, these observations indicate that Fz8 plays a critical role in the maintenance of quiescent LT-HSCs *in vivo*.

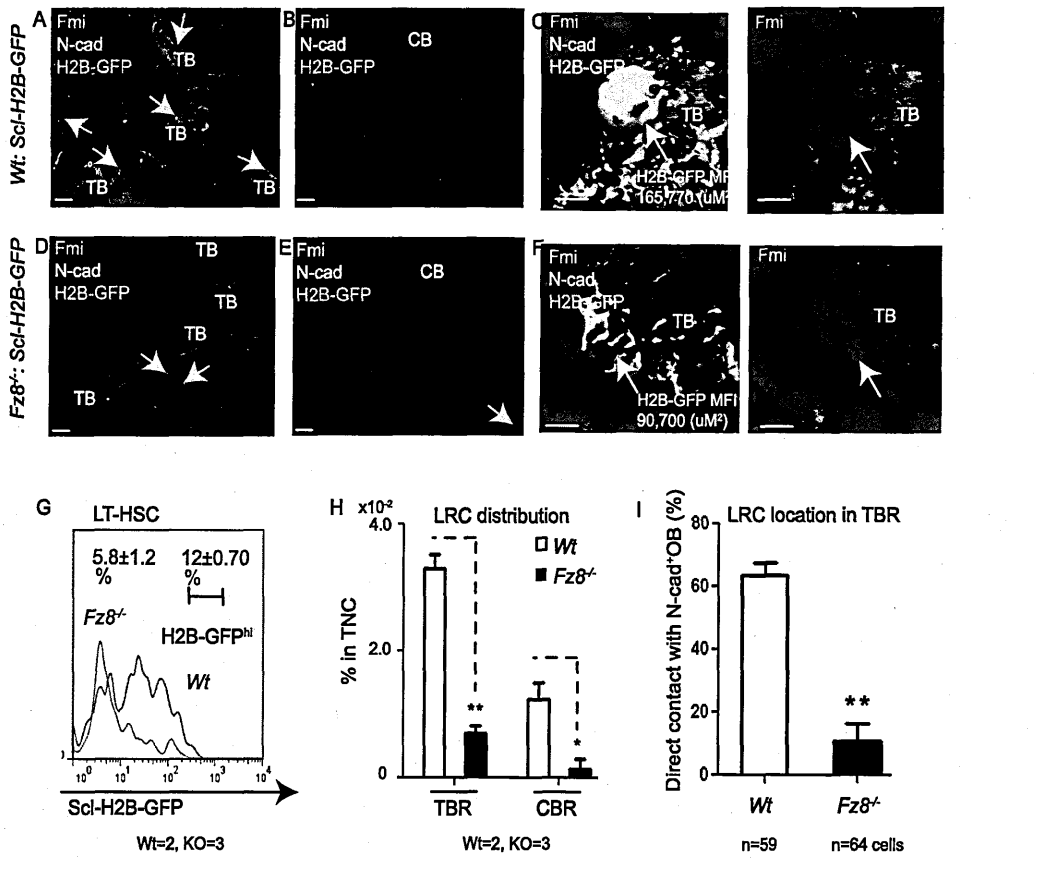

Figure 7-3 Label retention of *Fz8*$^{-/-}$ HSC

(A-F) Immunostaining of Fmi, N-cad, and H2B-GFP^{hi} LRC in TBR and CBR from *Wt: Scl-H2B-GFP* (A-C) and *Fz8*$^{-/-}$*: Scl-H2B-GFP* (D-F). Scale bar is 20uM (A-B, D-E) or 5uM (C, F). High-resolution 3D image of Fmi protein localization (red arrow) in H2B-GFP^{hi} LRC (C, F). (G) Flow cytometric analysis of H2B-GFP signal in LT-HSCs from *Wt* and *Fz8*$^{-/-}$. (H) H2B-GFP^{hi} LRC distribution in TBR and CBR from *Wt* and *Fz8*$^{-/-}$. Wt=2, KO=3 mice. (I) Frequency of H2B-GFP^{hi} LRCs directly contacting with N-cad^{+}OBs in TBR of *Wt* and *Fz8*$^{-/-}$. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

Chapter 8. Frizzled8 maintains quiescent HSCs by suppressing NFAT-induced IFNγ expression and antagonizing canonical Wnt signaling

In this chapter, I will analyze the downstream molecular events of non-canonical Wnt signaling mediated by Fz8 in HSCs.

I investigated the underlying molecular mechanism by which Fmi and Fz8-mediated non-canonical Wnt signaling maintains quiescent LT-HSCs. Non-canonical Wnt5a ligand stimulates the Ca^{2+}-NFAT signals in embryonic cells (Huang et al., 2011; Saneyoshi et al., 2002) but downregulates the Ca^{2+}-NFAT signals in adult cells (Dejmek et al., 2006). It is also known that non-canonical Wnt5a suppresses canonical Wnt activity (Nemeth et al., 2007) (Figure 8-1A). To examine the function of non-canonical Wnt signaling in HSCs in this context, I observed that the intracellular Ca^{2+} level, as measured using a Ca^{2+} reporter (see Experimental Procedure), increased 2-fold in the *Fz8*$^{-/-}$ LT-HSCs (Figure 8-1B, D-E). Canonical Wnt target gene Axin2 was upregulated 6 times more in active HSCs (Flk2$^-$H2B-GFP$^-$LSK cells) than in quiescent HSCs (H2B-GFP^{hi} Flk2$^-$LSK cells) (Figure 8-1C). These observations indicate that non-canonical Wnt signaling mediated by Fz8 suppresses the Ca^{2+}-NFAT pathway as well as canonical Wnt signaling in HSCs.

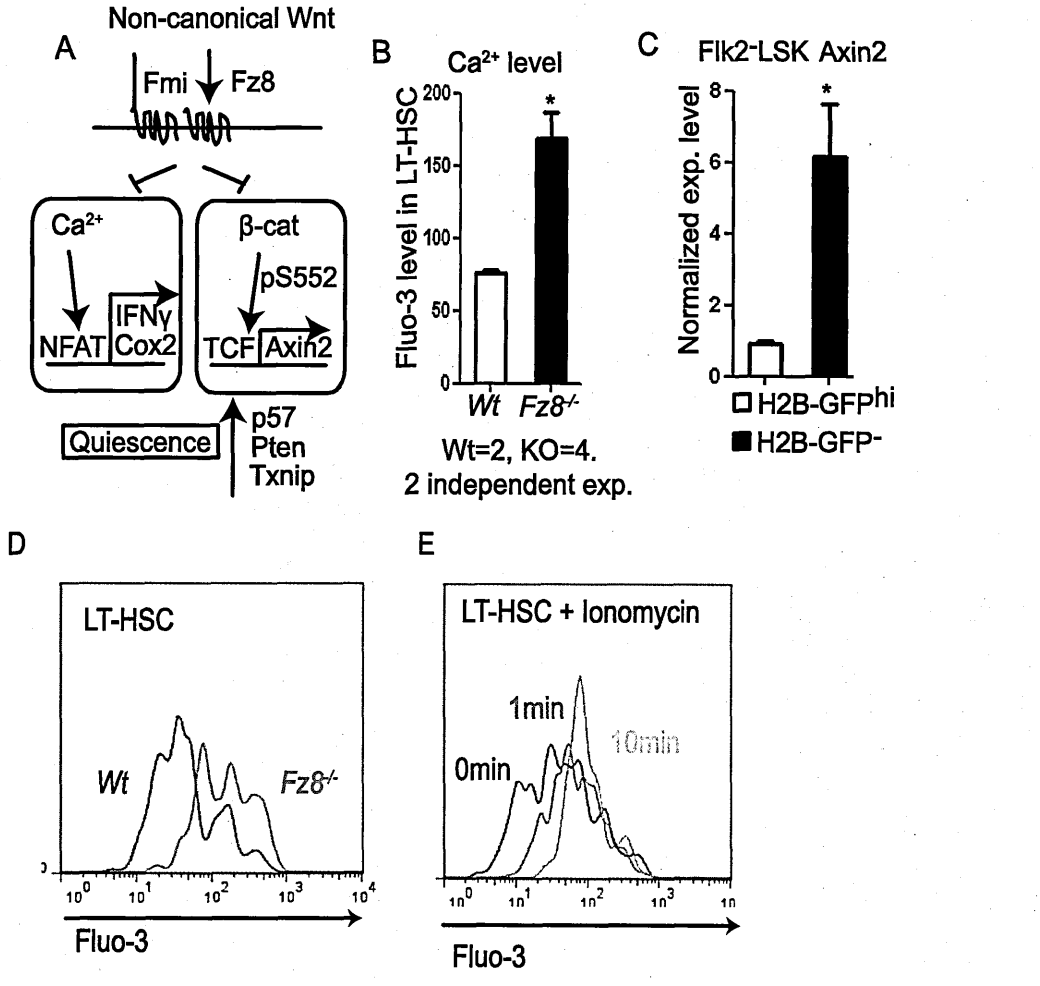

Figure 8-1 Canonical and non-canonical Wnt signaling in HSC

(A) Ca^{2+}-NFAT pathway and β-catenin pathway regulated by non-canonical Wnt signaling. (B) Intracellular Ca^{2+} level in LT-HSCs in *Wt* and *Fz8*$^{-/-}$. 2 independent experiments. (C) qRT-PCR analysis of *Axin2* in quiescent HSCs (Flk2$^-$H2B-GFP^{hi}LSK LRCs) and active HSCs (Flk2$^-$H2B-GFP$^-$LSK). (D) Fluo-3 intracellular Ca^{2+} analysis of LT-HSCs comparing *Fz8*$^{-/-}$ and *Wt*. (E) Positive control of Fluo-3 analysis with Ionomycin in LT-HSCs (0min, 1min and 10min post Ionomycin). (Modified from Sugimura et al., Cell 150, 351-365, 2012)

To confirm this, I observed in quiescent Fmi$^+$Fz8$^+$ HSCs (H2B-GFP^{hi} Flk2$^-$ LSK) that NFATc1 (shown to be highly expressed in LT-HSCs by RNA-seq) was mainly localized in the cytoplasm (Figure 8-2A, left panel). In contrast, however, in active Fmi$^-$Fz8$^-$HSCs (H2BGFP$^-$ Flk2$^-$LSK), NFATc1 was accumulated in the nucleus (Figure 8-2A, right panel). Statistically, only 4.3% of quiescent HSCs exhibited nuclear localized NFATc1 versus 87.7% of active HSCs (Figure 8-2B).

This observation indicates a correlation between cytoplasmic versus nuclear localization of NFAT and the quiescent versus active state of HSCs.

I further tested whether Fmi and Fz8 regulate NFAT protein localization in HSCs using knockdown analysis. In the scramble control, NFAT was mainly localized in the cytoplasm of infected LSK cells. In contrast, when either Fmi or Fz8 was knocked down, more than 80% of LSK cells showed nuclear accumulation of NFAT (Figure 8-2C-D). In addition, Fmi knockdown in OP9 cells induced NFAT nuclear translocation in LSKs that were co-cultured with OP9, suggesting that homophilic interaction of Fmi in adjacent cells regulates NFAT nuclear translocation.

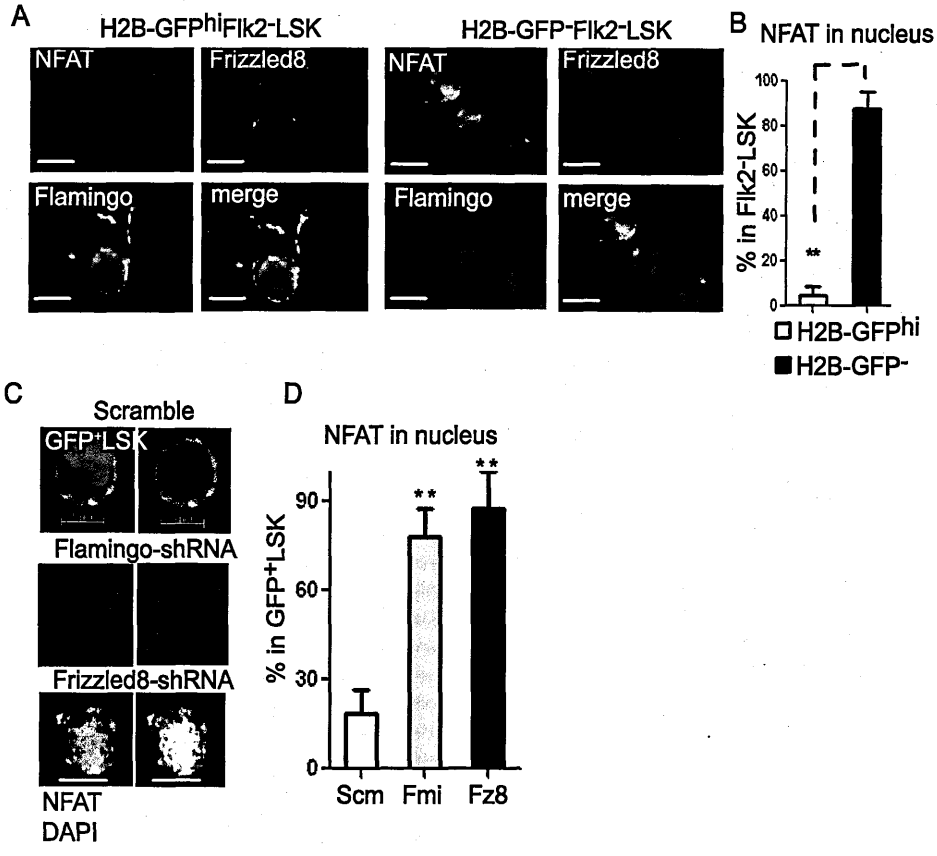

Figure 8-2 NFAT nuclear translocation in HSC

(A) Fmi, Fz8, and NFAT staining in sorted quiescent HSCs (Flk2$^-$H2B-GFP^{hi}LSK LRCs) and active HSCs (Flk2$^-$H2B-GFP$^-$LSK); NFAT (red), Fmi (white), Fz8 (green), and DAPI (blue). Scale bar is 5uM. (B) Percentage of HSCs with NFAT nuclear translocation. (C) *Fmi-, Fz8-* knockdown LSK cells stained with NFAT; NFAT (red), and DAPI (blue). (D) Percentage of cells with NFAT nuclear translocation. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

I then forced expression of a constitutive active (or nuclear localized) form of NFAT (CA-NFAT) (Monticelli and Rao, 2002) (Figure 8-3A) to test whether NFAT activates HSCs. Cell-cycle analysis of LSKs expressing CA-NFAT showed a decrease of quiescent HSCs and a substantial increase of cycling HSCs (Figure 8-3B-C), indicating that NFAT promotes HSC activation. I further confirmed this observation by showing that NFAT inhibitor could rescue the phenotype of a reduction in the G0-phase Flk2$^-$LSK HSCs (compare Figure 8-3B to 8-3D).

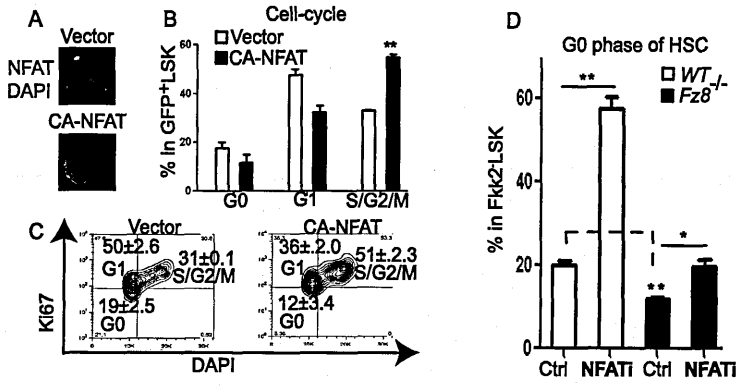

Figure 8-3 NFAT regulates HSC activation

(A) Immunostaining of NFAT with CA-NFAT and vector control transfected cells. (B-C) DNA content (DAPI) versus Ki67 staining of GFP^+LSK cells. Representative flow panels. Percentage of cells with G0, G1, and S/G2/M phases; vector control (white), and CA-NFAT (black). (D) Percentage of quiescent (G0 phase) $Flk2^-LSK$ comparing *Wt* and $Fz8^{-/-}$ with or without NFAT inhibitor (NFATi). (Modified from Sugimura et al., Cell 150, 351-365, 2012)

To further specify the downstream target genes of NFAT, I examined the genes involved in maintaining HSC quiescence, such as p57, Pten and Txnip (Jeong et al., 2009; Yilmaz et al., 2006b; Yoshihara et al., 2007; Zhang et al., 2006; Zou et al., 2011). I showed that these genes were overall downregulated to varying degrees in Fmi or Fz8 knockdown HSCs (Figure 8-4A).

Using qRT-PCR analysis of NFAT target genes, I found a 4-fold increase of IFNγ expression and a 1.8-fold increase of Cox2 in $Fmi^{-/-}$ LT-HSCs respectively (Figure 8-4B). I further observed a 3-fold increase of IFNγ expression in $Fz8^{-/-}$ LT-HSCs (Figure 8-4C), which is consistent with the observation that forced expression of CA-NFAT increased IFNγ by 2-fold in HSCs (Figure 8-4D). This upregulation of IFNγ expression could not be rescued by non-canonical Wnt5a ligand (Figure 8-4E), but could be rescued by NFAT inhibitor (Figure 8-4F). The results support that non-canonical Wnt5a-Fz8 signaling has a role in suppressing the NFAT-IFNγ pathway.

To examine the antagonization between canonical and non-canonical Wnt signaling, I conducted an *in vitro* culture experiment. Non-canonical Wnts

downregulated, whereas canonical Wnts upregulated IFNγ expression, and the latter showed a synergistic effect with *Fz8*$^{-/-}$ LT-HSCs (Figure 8-4G). All these results support an antagonization between canonical and non-canonical Wnt signaling to regulate downstream IFNγ expression.

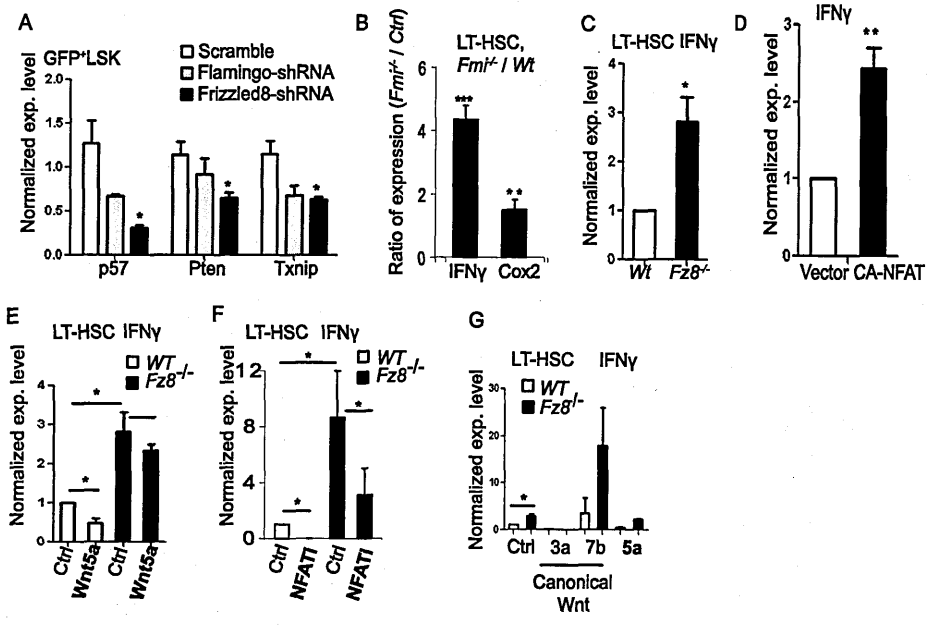

Figure 8-4 NFAT target genes in HSC

(A) qRT-PCR analysis of quiescence-related genes; scramble (white), Fmi-shRNA (gray), and Fz8-shRNA (black). Reactions were triplicated (hereafter). (B) qRT-PCR analysis of NFAT-target genes, *IFNγ* and *Cox2* in LT-HSCs comparing *Wt* and *Fmi*$^{-/-}$. (C) qRT-PCR of *IFNγ* in LT-HSCs comparing *Wt* and *Fz8*$^{-/-}$. (D) qRT-PCR for *IFNγ* of CA-NFAT infected HSCs. (E) qRT-PCR analysis of *IFNγ* in LT-HSCs cultured with Wnt5a. (F) qRT-PCR analysis of *IFNγ* in LT-HSCs comparing *Wt* and *Fz8*$^{-/-}$ with or without NFAT inhibitor (NFATi). (G) qRT-PCR analysis of *IFNγ* in LT-HSCs cultured with Wnt ligands. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

Mechanistically, non-canonical Wnt ligands suppress the Ca^{2+}-NFAT-IFNγ pathway. In contrast, canonical Wnt ligands and *Fz8*$^{-/-}$ promote NFAT nuclear translocation (Figure 8-5A, E-G, Figure 8-6A). I also examined the downstream event of canonical Wnt signaling using immunostaining of active β-catenin (β-cat-pS552) (He et al., 2007), TOP-Gal staining, Axin2-d2EGFP reporter, and canonical Wnt target Axin2 expression. I confirmed that canonical Wnts, loss of Fz8 or Fmi, all

increased nuclear-localized β-catenin-pS552, TOP-Gal staining, Axin2-d2EGFP level, in LT-HSCs (Figure 8-5B-D, Figure 8-6B-E). Taken together, all these observations indicate that non-canonical Wnt signaling via Fmi-Fz8 blocks the Ca^{2+}-NFAT-IFNγ pathway and canonical Wnt signaling.

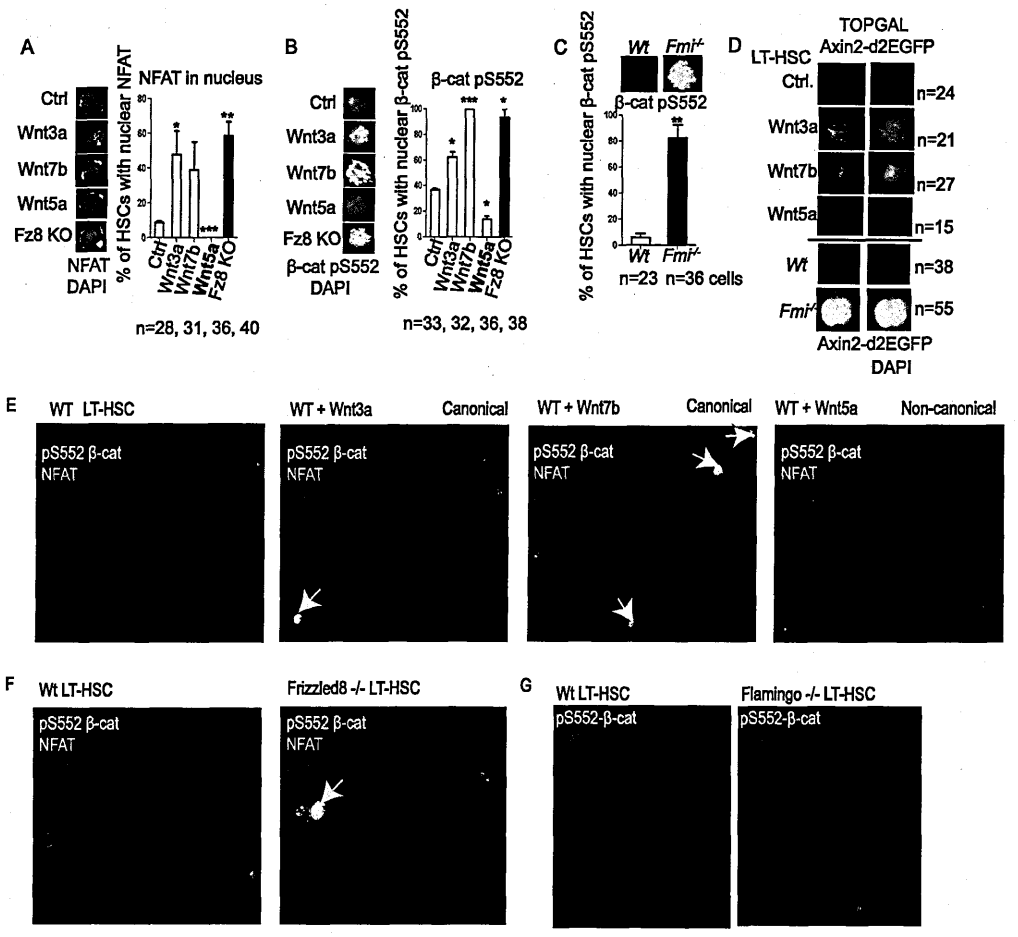

Figure 8-5 Analysis of canonical and non-canonical Wnt signaling in HSC

(A) NFAT immunostaining of LT-HSCs cultured with Wnt ligands. Percentage of NFAT nuclear translocation. n=28, 31, 36, 40 cells. (B) β-catenin-pS552 staining of LT-HSCs cultured with Wnt ligands. Percentage of HSCs with β-catenin-pS552 in nucleus. (C) β-catenin-pS552 staining of LT-HSCs from *Fmi*$^{-/-}$ and *Wt*. Percentage of HSCs with β-catenin-pS552 in nucleus. (D) TOP-GAL and Axin2-d2EGFP staining in LT-HSCs cultured with Wnt ligands or *Fmi*$^{-/-}$. n=24, 21, 27, 15, 38, 55 cells. (E) NFAT and β-catenin-pS552 staining of LT-HSCs cultured with Wnt ligands. Green arrows indicate cells with nuclear-localized β-catenin-pS552. (F) NFAT and β-catenin-pS552 staining of LT-HSCs from *Fz8*$^{-/-}$ and Wt. Green arrow indicates a cell with nuclear-localized β-catenin-pS552. (G) β-catenin-pS552 staining of LT-HSCs from *Fmi*$^{-/-}$ and Wt mice. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

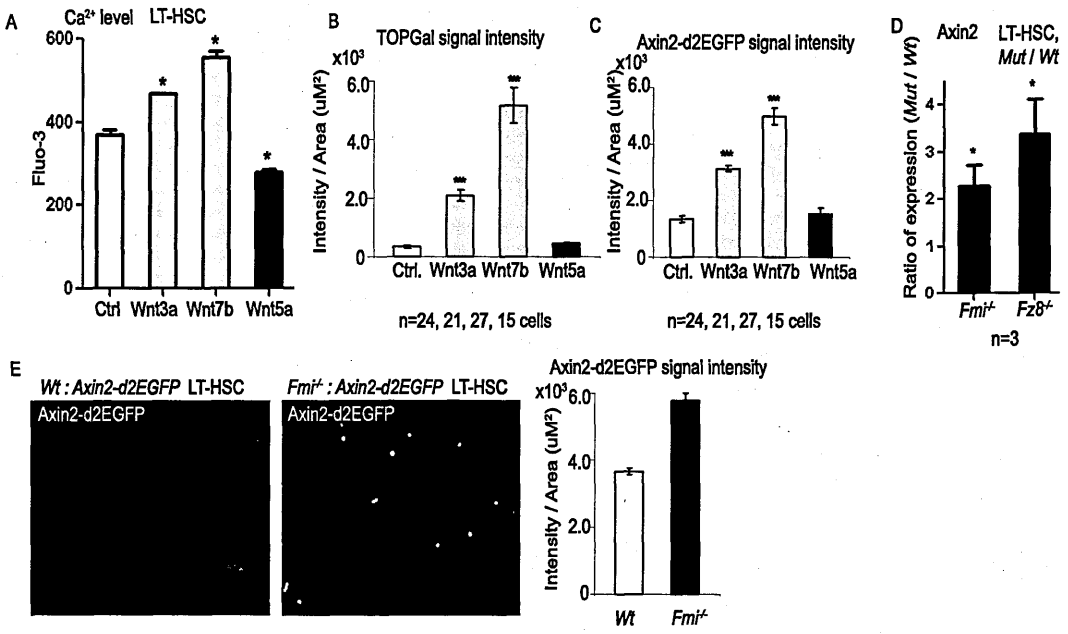

Figure 8-6 Analysis of canonical and non-canonical Wnt signaling in HSC (continued)

(A) Fluo-3 intracellular Ca2+ analysis of LT-HSCs cultured with Wnt ligands. (B-C) TOPGal staining (B) and Axin2-d2EGFP reporter analysis (C) of LT-HSCs cultured with Wnt ligands. n=24, 21, 27, 15 cells. (D) qRT-PCR for Axin2 in *Fmi*$^{-/-}$ and *Fz8*$^{-/-}$ compared to Wt. (E) d2EGFP reporter staining for Wt and *Fmi*$^{-/-}$ LT-HSCs. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

Chapter 9. General Discussion

In this work, I observed that a protein complex of Fmi and Fz8 co-localized in quiescent LT-HSCs that are predominantly located in TBR. Functionally, Fmi- and Fz8-mediated non-canonical Wnt signaling plays a critical role in maintaining quiescent LT-HSCs. Mechanistically, this non-canonical Wnt signaling suppresses HSC activation, in part through inhibiting the NFAT-IFNγ pathway, and in part through antagonizing canonical Wnt signaling during homeostasis. In response to stress, however, there is a decline of non-canonical Wnt signaling, accompanied by an increase of canonical Wnt signaling and activation of HSCs. Thus I provide strong evidence to distinguish the roles of non-canonical and canonical Wnt signaling in maintaining quiescent HSCs and in activating HSCs respectively.

9.1 Flamingo-Frizzled8-mediated non-canonical Wnt signaling, predominantly in the N-cad$^+$OB niche, is critical for maintaining quiescent long-term HSCs

I found a correlation between the expression of Fmi and Fz8 in quiescent LT-HSCs and their function in maintaining these HSCs. The remaining question was at which niche component did Fmi and Fz8 primarily function. Multiple HSC niche components have been reported to date, including OBs (particularly N-cad$^+$OBs), endothelial cells, Nestin-GFP$^+$ MSC-like cells, bipotential CAR (CXCL12 abundant reticular) cells, and Schwann cells (Calvi et al., 2003; Kiel et al., 2005; Mendez-Ferrer et al., 2010; Omatsu et al., 2010; Sugiyama et al., 2006; Yamazaki et al., 2011; Zhang et al., 2003a). The functional role of OBs was previously tested by Col2.3ΔTK-induced ablation of mature OB cells, and this resulted in a much delayed

loss of HSCs (Visnjic et al., 2004). Osteoclasts also influenced HSCs by indirectly regulating osteoblasts (Lymperi et al., 2011). In contrast, genetic ablation of Nestin^{+}MSCs and CAR cells caused a rapid mobilization of 50-60% of HSCs from BM to spleen. This was consistent with the distribution of 60% of HSCs in the sinusoidal perivascular niche (Kiel et al., 2005). Notably, the lost or mobilized HSCs were initially proliferating (or active) HSCs (Omatsu et al., 2010). These observations suggested that different niche components may form different microenvironments— quiescent versus active niches (Li and Clevers, 2010). The perivascular-localized Nestin-GFP^{+} and CAR cells, together with endothelial cells, most likely form an active niche, as they mainly support active HSCs (Butler et al., 2010). In contrast, the endosteal-localized N-cad^{+}OBs in TBR may form a quiescent niche. A previous report (Dominici et al., 2009) and my finding that N-cad^{+}OB is quiescent and drug-resistant show that N-cad^{+}OB is a very stable niche component even under stress (Figure 9A-B).

The model that N-cad^{+}OBs form a quiescent HSC niche is further supported by my observation that Fmi and Fz8 expressed at the interface between quiescent HSCs and N-cad^{+}OBs in TBR. Fmi homophilic adhesion has been shown to mediate 'contact inhibition' and transduce non-canonical Wnt signaling between neural axons (Kimura et al., 2006). I have shown that Fmi, in both HSCs and niche cells, regulates Fz8 distribution, consistent with the "contact inhibition" function of Fmi in maintaining the quiescent state of HSCs in the niche. Additionally, by comparing the expression of non-canonical Wnts and canonical Wnts/inhibitors, I found that endothelial cells expressed detectable levels of Wnt5b and that Nestin-GFP^{+} cells expressed limited canonical Wnts, such as Wnt10b. In contrast, N-cad^{+}OBs

expressed the highest levels of Wnt inhibitors that suppress canonical Wnt signaling in homeostasis. Although, both Nestin-GFP^{+} cells and N-cad^{+}OBs expressed several non-canonical Wnts, N-cad^{+}OBs predominantly expressed additional Wnt6 and Wnt16. Notably, Wnt16 was recently shown to be critical in inducing HSC generation from endothelial progenitor cells during fetal development of zebrafish (Clements et al., 2011). All these observations support that N-cad^{+}OBs maintain a quiescent niche with dominant non-canonical Wnt signaling and simultaneously suppress canonical Wnt signaling in homeostasis. This dominant expression of non-canonical Wnts is consistent with the presence of Fmi-Fz8 complex in the N-cad^{+}OB niche (Figure 9B).

9.2 Flamingo-Frizzled8-mediated non-canonical Wnt signaling suppresses Ca^{2+}-NFAT-IFNγ-pathway and antagonizes canonical Wnt signaling, thereby preventing HSCs from activation

Recently, non-canonical Wnt signaling was shown to be involved in HSC development (Clements et al., 2011; Heinonen et al., 2011; Louis et al., 2008) and in HSC maintenance *in vitro* culture (Murdoch et al., 2003; Nemeth et al., 2007). The underlying mechanism, however, was undefined. The downstream pathways of non-canonical Wnt signaling have diverse functions: forming planar cell polarity, blocking Ca^{2+}-NFAT nuclear translocation, and suppressing β-catenin activity. In this work, I demonstrated that Fmi-Fz8 mediated non-canonical Wnt signaling indeed suppresses nuclear translocation of NFAT. NFAT was previously shown to maintain hair follicle stem cells through suppression of Cdk4 (Horsley et al., 2008)

and to play an important role in hematopoiesis (Muller et al., 2009). In HSCs, I identified that NFAT stimulates IFNγ. Another niche component, Treg cell, also expresses IFNγ post 5FU. I do not exclude that other stromal cells as well might express IFNγ. While IFNs play a critical role in activating HSCs (Baldridge et al., 2010; Essers et al., 2009), Fmi- and Fz8-mediated non-canonical Wnt signaling maintains quiescence, partially through downregulation of IFNγ expression (Figure 9C). I further demonstrated that culturing HSCs with Wnt5a inhibits IFNγ expression. This inhibitory effect ended when Fz8 was knocked out, further supporting the critical role of Fz8-mediated non-canonical Wnt signaling to suppress the Ca^{2+}-NFAT-IFNγ pathway. In addition, I showed that Fmi-Fz8-mediated non-canonical Wnt signaling antagonizes canonical Wnt signaling, which is consistent with a previous report that Wnt5a and Fz8-mediated non-canonical Wnt signaling inhibited canonical Wnt signaling (Mikels and Nusse, 2006; Nemeth et al., 2007) (Figure 9C).

9.3 Non-canonical versus canonical Wnt signaling have distinguished roles respectively in maintenance vs. activation of HSCs

Wnt signaling, particularly the canonical pathway through activation of β-catenin, has been shown across several species to be prominent in regulating stem cell self-renewal in both embryonic and adult stem cells (Blanpain and Fuchs, 2009; Reya et al., 2003; van de Wetering et al., 2002; Ying et al., 2008). The role of canonical Wnt signaling in HSC maintenance, though, is debatable. For example, forced expression of Dkk1 in osteoblasts suppressed canonical Wnt signaling in

HSCs, accompanied by a decrease in quiescent HSCs (Fleming et al., 2008). However, other studies showed that Dkk1-transgenic mice had a defect in trabecular bone formation (Guo et al., 2010; Li et al., 2006). This is consistent with the role of canonical Wnt signaling in osteogenesis during trabecular bone formation. For example, absence of β-catenin in osteoblasts led to a defect in trabecular bone formation, resulting in a decrease of quiescent HSCs (Nemeth et al., 2009). Therefore, it is most likely that Dkk1 directly affects the osteoblastic niche, and thus indirectly influences the resident quiescent HSCs. A similar observation was reported that inactivation in bone of Sfrp1, a soluble canonical Wnt inhibitor, resulted in the decrease of HSCs (Renstrom et al., 2009). Another example is that forced expression of Wif1 in osteoblasts reduced quiescent HSCs. Although overexpression of Wif1 in a transgenic model did not alternate bone architecture, Wif1 inhibited non-canonical Wnt signaling (Hsieh et al., 1999) and thus increased canonical Wnt signaling (evidenced by increased Wnt3a) in HSCs (Schaniel et al., 2011).

The association of non-canonical Wnt signaling with N-cad^{+}OBs provides insight to reconciling previous contradictory observations.

First, ablation of Nestin-GFP^{+} cells or CAR-cells leads to a rapid loss of a portion of HSCs. This observation can be explained by the role of these niche components to support primarily the active HSC subpopulation (Omatsu et al., 2010). In contrast, ablation of mature osteoblasts induced by Col2.3ΔTK (thymidine kinase) results in a much delayed reduction of HSCs, which is consistent with the role of this niche component to support the long-term quiescent HSC subpopulation (Visnjic et al., 2004). This is because loss of quiescent HSCs often does not have an immediate influence on hematopoiesis, as the active population still supports

hematopoiesis. Furthermore, reduction in quiescent HSCs often leads to an increase in cycling HSCs, and therefore the immunophenotypic measurement of total HSCs is not affected in the short term. The observation that there is a subsequent recovery of HSCs following deletion of mature OBs induced by Col2.3ΔTK (Visnjic et al., 2004) can be explained by Col2.3 being mainly expressed in mature OBs. Therefore, Col2.3 induced TK cannot efficiently target N-cad$^+$OBs that enrich quiescent osteoprogenitors (Xie et al., 2009). The untargeted N-cad$^+$OBs can facilitate recovery of osteogenesis initially and then subsequent HSC recovery.

Second, Fmi, as an atypical cadherin family molecule, mediates a homophilic interaction between N-cad$^+$OBs and HSCs, and provides a redundant role as that of N-cadherin in mediating HSC-niche interaction (Figure 9C). This may account for the subtle phenotype seen in the N-cad conditional KO model (Kiel et al., 2008).

N-cad$^+$OBs may not just play a passive role in maintaining quiescent HSCs, as they have been implicated to facilitate HSC expansion in response to irradiation-induced BM damage (Dominici et al., 2009). Another report also showed the correlation between the number of HSCs and N-cad$^+$OBs, but not mature OBs (Lymperi et al., 2008). Consistent with this observation, I found that in response to 5FU-induced BM damage, non-canonical Wnt signaling in N-cad$^+$OBs was downregulated (Figure 9D-E). This microenvironmental change was further supported by specific and strong stimulation of IFNγ by Wnt7b *in vitro*. In this experiment, Wnt7b also promoted an increase in the active form (β-cat-pS552) of β-catenin in the HSC nucleus, clearly indicating antagonizing roles between canonical and non-canonical Wnt signaling. The attenuation of non-canonical Wnt signaling may coordinate with other niche components with different activation signals (such

as SCF etc.) to facilitate HSC activation and subsequent expansion. This new finding may help reconcile the contradictory reports regarding canonical Wnt signaling in HSCs. In a previous study, for example, inactivation of β-catenin did not affect homeostatic HSCs (Cobas et al., 2004). This is because canonical Wnt signaling is not prominent during homeostasis, as revealed in my study. On the other hand, inactivation of β-catenin during embryonic and fetal stages indeed affected hematopoiesis, due to involvement of canonical Wnt signaling in HSC expansion. Transgenic expression of stabilized β-catenin leading to HSC reduction (Kirstetter et al., 2006; Scheller et al., 2006) seems not to support the role of canonical Wnt signaling in HSC self-renewal and expansion. However, this observation can be explained by our recent report that constitutive expression of β-catenin in HSCs induced apoptosis. Only in coordination with PI3K/Akt (or Bcl2) signaling, can HSCs be expanded with canonical Wnt signaling (Perry et al., 2011b; Reya et al., 2003). My study provides novel evidence to distinguish the respective roles of non-canonical versus canonical Wnt signaling in maintenance versus activation and expansion of HSCs.

9.4. Expression of Flamingo subtypes in HSCs

Flamingo family has 3 subtypes, Celsr1, 2, and 3. In this study, I found that Flamingo/Celsr2 is expressed in quiescent label-retaining HSCs in the endosteal zone of TBR. I also found Celsr1 was not expressed in HSCs. In contrast, Celsr3 is expressed in mobilized HSCs following 5FU-mediated activation in blood vessels. This indicates Celsr3 may regulate HSC maintenance of mobilized HSCs which have been reported in quiescent state (Morrison et al., 1997). In the future, it will be interesting to test whether deletion of Celsr3 affects HSCs during circulation.

9.5. Canonical and non-canonical Wnt signaling

Wnt signaling can be separated into canonical and non-canonical signaling. Which signaling is to be used depends on the tissue types that express different types of Frizzleds and other co-receptors. For example, Wnt5a activates non-canonical Wnt signaling in the presence of Ror2 co-receptor. In contrast, Wnt5a activates canonical Wnt signaling in the presence of Frizzled4 (Mikels and Nusse, 2006). In my study using TOP-Gal and Axin2-d2EGFP reporter models, I have confirmed that Wnt5a activates non-canonical Wnt and suppresses canonical Wnt signaling.

The structure of Wnt and Frizzled recognition remains unclear. A recent report showed the 3D structure of Xenopus Wnt8 and mouse Fz8 binding (Janda et al., 2012). According to the report, Wnt structure resembles a "hand" with "thumb" and "index" fingers extended to grasp Frizzled8 cysteine-rich domain at two distinct binding sites. This observation suggests that the different biding sites of Wnts and Frizzleds may fine-tune the downstream pathways with co-receptors, thus determining canonical or non-canonical Wnt signaling.

9.6. Wnt signaling and BMP signaling in determination of quiescent vs. active niches

Our lab previously showed that BMP signaling in the endosteal zone regulates HSC and ISC maintenance (He et al., 2004b; Zhang et al., 2003a). In contrast, canonical Wnt signaling through β-catenin promotes proliferation of HSCs and ISCs (He et al., 2007; Perry et al., 2011a). These observations indicate "Yin-Yang control" of stem cells by canonical Wnt signaling and BMP signaling. In my study, non-canonical Wnt signaling has been identified as another signaling to maintain quiescent HSCs. My preliminary data showed that BMP4 is expressed in

the TBR; in contrast, BMP inhibitor Noggin is expressed in the compact bone region. A recent report showed that BMP4 and BMP7 induce osteoblast differentiation of MSCs via histone demethylases KDM4B and KDM6B which activate the chromatin region (Ye et al., 2012). These observations suggest that BMP signaling may form the endosteal zone of TBR, where non-canonical Wnt signaling facilitates a local control of quiescent HSCs adjacent to N-cad^{+}OBs. After stress, non-canonical Wnt signaling switches to canonical Wnt signaling to allow HSC activation. Canonical Wnt signaling determines the active HSC niche.

9.7. Dynamic regulation of Wnt signaling balance during development and aging

It is very interesting to consider the state of Wnt signaling balance in the niches in fetal stage BM and adult mice. Since fetal HSCs are proliferating and a portion of adult HSCs are quiescent, it seems that the balance of canonical and non-canonical Wnt signaling may be different between fetal and adult stages. Adult HSCs are more deeply quiescent which are difficult to activate. This may suggest non-canonical Wnt signaling is very high in the adult HSC niche. It is intriguing to see the level of non-canonical Wnts expression in adult niche cells.

Recently, Eaves and colleagues studied α-HSCs (lymphoid-deficient) and β-HSCs (balanced-lineage) and revealed that α-HSCs became predominant in adult mice, which explains why adult HSCs are more committed to myeloid and deficient for lymphoid lineage (Benz et al., 2012). Indeed, their data showed that α-HSCs and β-HSCs interconverted at secondary transplantation, which suggests HSCs can be redistributed to different niches so that HSC states can be extrinsically influenced. It

is intriguing to see whether this switch is correlated with canonical versus non-canonical Wnt signaling in association with niches.

9.8. Future direction

My study has revealed that the balance of Wnt signaling can regulate different populations of HSCs in anatomically distinct niches. Quiescent HSCs are adjacent with N-cad^{+}OBs in the TBR endosteal zone. This population functions as a reserve population and is maintained by non-canonical Wnt signaling. Under stress, the endosteal zone switches to canonical Wnt signaling to activate HSCs. Normally, active HSCs are located in the perivascular zone.

So far, the field has focused on canonical Wnt signaling, with fewer studies focused on non-canonical Wnt signaling in HSCs. My study proposed the model in which canonical and non-canonical Wnt signaling have opposite functions to regulate HSCs. Considering functional cross-talk of canonical and non-canonical Wnt signaling and their opposing effect, it is critical to clarify canonical and non-canonical Wnt signaling when we investigate Wnt signaling in HSCs.

In recent years, the HSC niche field has made remarkable progress and also has been very controversial. This is partly because HSCs reside in different locations. Label-retaining quiescent HSCs are in the endosteal zone with N-cad^{+}OBs (Zhang et al., 2003a); CD150^{+} CD48^{-} HSCs are in the perivascular and sinusoidal zones (Kiel et al., 2005; Mendez-Ferrer et al., 2010). The debate is ongoing regarding which is the "real" HSC niche. This controversy can be reconciled by a new model that HSCs may include both active and quiescent HSCs. The former is responsible for daily production of blood cells, whereas the latter is a reserve population that rarely enters

cell-cycle, but can be triggered to replenish lost active HSCs when needed (Li and Clevers, 2010; Wilson et al., 2008).

The remaining questions related to my study are how the balance of Wnt signaling is regulated in different niches, and whether and how active HSCs revert to quiescent HSCs (Wilson et al., 2008).

This thesis study showed the different and sequential process of HSC activation in anatomically and molecularly distinct zones, and the related different types of Wnt signaling. In order to have a systematic and comprehensive understanding of the niche network and the associated signaling, it will be important to investigate not only just one type of niche or signaling, but also multiple niches and both canonical and non-canonical Wnt signaling.

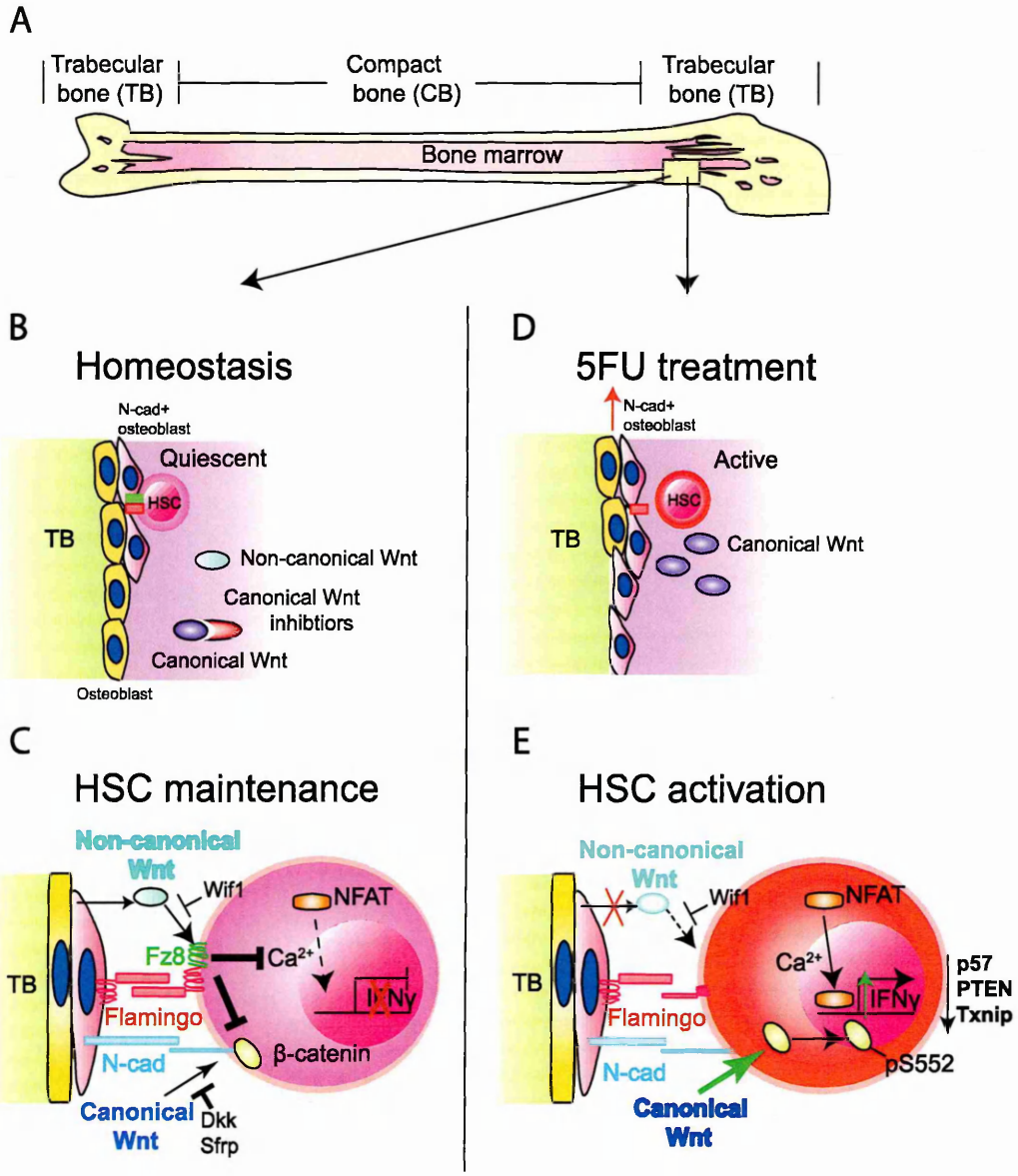

Figure 9-1 Model of non-canonical Wnt signaling maintaining HSC in the niche

(A) Sagittal section of femur indicating TBR and CBR. (B) HSC maintenance during homeostasis. Within TBR, a portion of quiescent LT-HSCs are adhered to N-cad^{+}OBs that maintain dominant non-canonical Wnt signals and suppress canonical Wnt signaling by their inhibitors. Fmi (red box in B) restricts Fz8 (green box in B) at the interface between quiescent HSCs and N-cad^{+}OBs, engaging interaction with local non-canonical Wnt ligands from N-cad^{+}OBs. (C) Fz8 suppresses Ca2+-NFAT nuclear translocation and NFAT-dependent IFNγ expression. N-cad tethers β-catenin, inhibiting canonical Wnt signaling. In addition, Fmi and Fz8 antagonize canonical Wnt signaling. Therefore, non-canonical Wnt signaling is predominant in quiescent HSCs. (D) Post 5FU treatment, canonical Wnt ligand (e.g. Wnt7b) expression is upregulated. In addition, the expression of non-canonical Wnt ligands (Wnt11 and Wnt16) and canonical Wnt inhibitors are declined. HSCs reduce both Flamingo and Fz8 expression. N-cad^{+}OBs reduce Fz8 expression. (E) Decrease in Fmi-Fz8 mediated non-canonical Wnt signaling resulted in NFAT-induced IFNγ expression; and increase in Wnt7b led to release of β-catenin to cytoplasm and further into nucleus, thus together promoting HSC activation. (Modified from Sugimura et al., Cell 150, 351-365, 2012)

REFERENCES

Ahumada, A, Slusarski, DC, Liu, X, Moon, RT, Malbon, CC, Wang, HY (2002) Signaling of rat Frizzled-2 through phosphodiesterase and cyclic GMP. Science *298*, 2006-2010.

Akashi, K, He, X, Chen, J, Iwasaki, H, Niu, C, Steenhard, B, Zhang, J, Haug, J, Li, L (2003) Transcriptional accessibility for genes of multiple tissues and hematopoietic lineages is hierarchically controlled during early hematopoiesis. Blood *101*, 383-389.

Alonso, L, Fuchs, E (2003a) Stem cells in the skin: waste not, Wnt not. Genes Dev *17*, 1189-1200.

Alonso, L, Fuchs, E (2003b) Stem cells of the skin epithelium. Proc Natl Acad Sci U S A *100 Suppl 1*, 11830-11835.

Arai, F, Hirao, A, Ohmura, M, Sato, H, Matsuoka, S, Takubo, K, Ito, K, Koh, GY, Suda, T (2004) Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the BM niche. Cell *118*, 149-161.

Arron, JR, Winslow, MM, Polleri, A, Chang, CP, Wu, H, Gao, X, Neilson, JR, Chen, L, Heit, JJ, Kim, SK, Yamasaki, N, Miyakawa, T, Francke, U, Graef, IA, Crabtree, GR (2006) NFAT dysregulation by increased dosage of DSCR1 and DYRK1A on chromosome 21. Nature *441*, 595-600.

Baksh, D, Tuan, RS (2007) Canonical and non-canonical Wnts differentially affect the development potential of primary isolate of human BM MSCs. Journal of cellular physiology *212*, 817-826.

Baldridge, MT, King, KY, Boles, NC, Weksberg, DC, Goodell, MA (2010) Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. Nature *465*, 793-797.

Beall, SA, Boekelheide, K, Johnson, KJ (2005) Hybrid GPCR/cadherin (Celsr) proteins in rat testis are expressed with cell type specificity and exhibit differential Sertoli cell-germ cell adhesion activity. Journal of andrology *26*, 529-538.

Benveniste, P, Frelin, C, Janmohamed, S, Barbara, M, Herrington, R, Hyam, D, Iscove, NN Intermediate-term hematopoietic stem cells with extended but time-limited reconstitution potential. Cell Stem Cell *6*, 48-58.

Benz, C, Copley, MR, Kent, DG, Wohrer, S, Cortes, A, Aghaeepour, N, Ma, E, Mader, H, Rowe, K, Day, C, Treloar, D, Brinkman, RR, Eaves, CJ (2012) Hematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. Cell stem cell *10*, 273-283.

Bhanot, P, Fish, M, Jemison, JA, Nusse, R, Nathans, J, Cadigan, KM (1999) Frizzled and Dfrizzled-2 function as redundant receptors for Wingless during Drosophila embryonic development. Development *126*, 4175-4186.

Bhat, RA, Stauffer, B, Della Pietra, A, Bodine, PV (2010) Wnt3-frizzled 1 chimera as a model to study canonical Wnt signaling. J Cell Biochem *109*, 876-884.

Blankesteijn, WM, Essers-Janssen, YP, Ulrich, MM, Smits, JF (1996) Increased expression of a homologue of drosophila tissue polarity gene "frizzled" in left

ventricular hypertrophy in the rat, as identified by subtractive hybridization. J Mol Cell Cardiol *28*, 1187-1191.

Blanpain, C, Fuchs, E (2009) Epidermal homeostasis: a balancing act of stem cells in the skin. Nature reviews *10*, 207-217.

Blanpain, C, Lowry, WE, Geoghegan, A, Polak, L, Fuchs, E (2004) Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell *118*, 635-648.

Boisset, JC, van Cappellen, W, Andrieu-Soler, C, Galjart, N, Dzierzak, E, Robin, C (2010) In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. Nature *464*, 116-120.

Boland, GM, Perkins, G, Hall, DJ, Tuan, RS (2004) Wnt 3a promotes proliferation and suppresses osteogenic differentiation of adult human MSCs. J Cell Biochem *93*, 1210-1230.

Boutros, M, Paricio, N, Strutt, DI, Mlodzik, M (1998) Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. Cell *94*, 109-118.

Boyden, LM, Mao, J, Belsky, J, Mitzner, L, Farhi, A, Mitnick, MA, Wu, D, Insogna, K, Lifton, RP (2002) High bone density due to a mutation in LDL-receptor-related protein 5. The New England journal of medicine *346*, 1513-1521.

Butler, JM, Nolan, DJ, Vertes, EL, Varnum-Finney, B, Kobayashi, H, Hooper, AT, Seandel, M, Shido, K, White, IA, Kobayashi, M, Witte, L, May, C, Shawber, C, Kimura, Y, Kitajewski, J, Rosenwaks, Z, Bernstein, ID, Rafii, S (2010) Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. Cell stem cell *6*, 251-264.

Calvi, LM, Adams, GB, Weibrecht, KW, Weber, JM, Olson, DP, Knight, MC, Martin, RP, Schipani, E, Divieti, P, Bringhurst, FR, Milner, LA, Kronenberg, HM, Scadden, DT (2003) Osteoblastic cells regulate the haematopoietic stem cell niche. Nature *425*, 841-846.

Challen, GA, Goodell, MA (2008) Promiscuous expression of H2B-GFP transgene in hematopoietic stem cells. PloS one *3*, e2357.

Chang, J, Sonoyama, W, Wang, Z, Jin, Q, Zhang, C, Krebsbach, PH, Giannobile, W, Shi, S, Wang, CY (2007) Noncanonical Wnt-4 signaling enhances bone regeneration of MSCs in craniofacial defects through activation of p38 MAPK. The Journal of biological chemistry *282*, 30938-30948.

Chen, CM, Struhl, G (1999) Wingless transduction by the Frizzled and Frizzled2 proteins of Drosophila. Development *126*, 5441-5452.

Chen, WS, Antic, D, Matis, M, Logan, CY, Povelones, M, Anderson, GA, Nusse, R, Axelrod, JD (2008) Asymmetric homotypic interactions of the atypical cadherin flamingo mediate intercellular polarity signaling. Cell *133*, 1093-1105.

Christian, JL, Gavin, BJ, McMahon, AP, Moon, RT (1991) Isolation of cDNAs partially encoding four Xenopus Wnt-1/int-1-related proteins and characterization of their transient expression during embryonic development. Dev Biol *143*, 230-234.

Ciani, L, Boyle, KA, Dickins, E, Sahores, M, Anane, D, Lopes, DM, Gibb, AJ, Salinas, PC (2011) Wnt7a signaling promotes dendritic spine growth and synaptic strength through Ca(2)/Calmodulin-dependent protein kinase II. Proceedings of the National Academy of Sciences of the United States of America *108*, 10732-10737.

Clements, WK, Kim, AD, Ong, KG, Moore, JC, Lawson, ND, Traver, D (2011) A somitic Wnt16/Notch pathway specifies haematopoietic stem cells. Nature *474*, 220-224.

Cobas, M, Wilson, A, Ernst, B, Mancini, SJ, MacDonald, HR, Kemler, R, Radtke, F (2004) Beta-catenin is dispensable for hematopoiesis and lymphopoiesis. The Journal of experimental medicine *199*, 221-229.

Cotsarelis, G, Sun, TT, Lavker, RM (1990) Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell *61*, 1329-1337.

De Calisto, J, Araya, C, Marchant, L, Riaz, CF, Mayor, R (2005) Essential role of non-canonical Wnt signalling in neural crest migration. Development (Cambridge, England) *132*, 2587-2597.

Deininger, M, Buchdunger, E, Druker, BJ (2005) The development of imatinib as a therapeutic agent for chronic myeloid leukemia. Blood *105*, 2640-2653.

Dejmek, J, Safholm, A, Kamp Nielsen, C, Andersson, T, Leandersson, K (2006) Wnt-5a/Ca2+-induced NFAT activity is counteracted by Wnt-5a/Yes-Cdc42-casein kinase 1alpha signaling in human mammary epithelial cells. Molecular and cellular biology *26*, 6024-6036.

Devenport, D, Fuchs, E (2008) Planar polarization in embryonic epidermis orchestrates global asymmetric morphogenesis of hair follicles. Nature cell biology *10*, 1257-1268.

Ding, L, Saunders, TL, Enikolopov, G, Morrison, SJ (2012) Endothelial and perivascular cells maintain haematopoietic stem cells. Nature *481*, 457-462.

Dominici, M, Rasini, V, Bussolari, R, Chen, X, Hofmann, TJ, Spano, C, Bernabei, D, Veronesi, E, Bertoni, F, Paolucci, P, Conte, P, Horwitz, EM (2009) Restoration and reversible expansion of the osteoblastic hematopoietic stem cell niche after marrow radioablation. Blood *114*, 2333-2343.

Drouet, M, Mourcin, F, Grenier, N, Delaunay, C, Mayol, JF, Lataillade, JJ, Peinnequin, A, Herodin, F (2005) MSCs rescue CD34+ cells from radiation-induced apoptosis and sustain hematopoietic reconstitution after coculture and cografting in lethally irradiated baboons: is autologous stem cell therapy in nuclear accident settings hype or reality? BM Transplant *35*, 1201-1209.

Elmslie, FV, Vivian, AJ, Gardiner, H, Hall, C, Mowat, AP, Winter, RM (1995) Alagille syndrome: family studies. J Med Genet *32*, 264-268.

Ema, H, Morita, Y, Yamazaki, S, Matsubara, A, Seita, J, Tadokoro, Y, Kondo, H, Takano, H, Nakauchi, H (2006) Adult mouse hematopoietic stem cells: purification and single-cell assays. Nature protocols *1*, 2979-2987.

Essers, MA, Offner, S, Blanco-Bose, WE, Waibler, Z, Kalinke, U, Duchosal, MA, Trumpp, A (2009) IFNalpha activates dormant haematopoietic stem cells in vivo. Nature.

Etienne-Manneville, S, Hall, A (2001) Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. Cell *106*, 489-498.

Etienne-Manneville, S, Hall, A (2003) Cdc42 regulates GSK-3beta and adenomatous polyposis coli to control cell polarity. Nature *421*, 753-756.

Flaherty, MP, Abdel-Latif, A, Li, Q, Hunt, G, Ranjan, S, Ou, Q, Tang, XL, Johnson, RK, Bolli, R, Dawn, B (2008) Noncanonical Wnt11 signaling is sufficient to induce cardiomyogenic differentiation in unfractionated BM mononuclear cells. Circulation *117*, 2241-2252.

Fleming, HE, Janzen, V, Lo Celso, C, Guo, J, Leahy, KM, Kronenberg, HM, Scadden, DT (2008) Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. Cell stem cell *2*, 274-283.

Florian, MC, Dorr, K, Niebel, A, Daria, D, Schrezenmeier, H, Rojewski, M, Filippi, MD, Hasenberg, A, Gunzer, M, Scharffetter-Kochanek, K, Zheng, Y, Geiger, H (2012) Cdc42 activity regulates hematopoietic stem cell aging and rejuvenation. Cell stem cell *10*, 520-530.

Fotaki, V, Dierssen, M, Alcantara, S, Martinez, S, Marti, E, Casas, C, Visa, J, Soriano, E, Estivill, X, Arbones, ML (2002) Dyrk1A haploinsufficiency affects viability and causes developmental delay and abnormal brain morphology in mice. Mol Cell Biol *22*, 6636-6647.

Foudi, A, Hochedlinger, K, Van Buren, D, Schindler, JW, Jaenisch, R, Carey, V, Hock, H (2008) Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. Nature biotechnology.

Friedenstein, AJ, Deriglasova, UF, Kulagina, NN, Panasuk, AF, Rudakowa, SF, Luria, EA, Ruadkow, IA (1974) Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol *2*, 83-92.

Friedenstein, AJ, Gorskaja, JF, Kulagina, NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol *4*, 267-274.

Friedenstein, AJ, Ivanov-Smolenski, AA, Chajlakjan, RK, Gorskaya, UF, Kuralesova, AI, Latzinik, NW, Gerasimow, UW (1978) Origin of BM stromal mechanocytes in radiochimeras and heterotopic transplants. Exp Hematol *6*, 440-444.

Fuentes, JJ, Genesca, L, Kingsbury, TJ, Cunningham, KW, Perez-Riba, M, Estivill, X, de la Luna, S (2000) DSCR1, overexpressed in Down syndrome, is an inhibitor of calcineurin-mediated signaling pathways. Hum Mol Genet *9*, 1681-1690.

Fujisaki, J, Wu, J, Carlson, AL, Silberstein, L, Putheti, P, Larocca, R, Gao, W, Saito, TI, Lo Celso, C, Tsuyuzaki, H, Sato, T, Cote, D, Sykes, M, Strom, TB, Scadden, DT,

Lin, CP (2011) In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. Nature *474*, 216-219.

Fukuda, T, Chen, L, Endo, T, Tang, L, Lu, D, Castro, JE, Widhopf, GF, 2nd, Rassenti, LZ, Cantwell, MJ, Prussak, CE, Carson, DA, Kipps, TJ (2008) Antisera induced by infusions of autologous Ad-CD154-leukemia B cells identify ROR1 as an oncofetal antigen and receptor for Wnt5a. Proc Natl Acad Sci U S A *105*, 3047-3052.

Gelebart, P, Anand, M, Armanious, H, Peters, AC, Dien Bard, J, Amin, HM, Lai, R (2008) Constitutive activation of the Wnt canonical pathway in mantle cell lymphoma. Blood *112*, 5171-5179.

Giles, RH, van Es, JH, Clevers, H (2003) Caught up in a Wnt storm: Wnt signaling in cancer. Biochim Biophys Acta *1653*, 1-24.

Goss, AM, Tian, Y, Tsukiyama, T, Cohen, ED, Zhou, D, Lu, MM, Yamaguchi, TP, Morrisey, EE (2009) Wnt2/2b and beta-catenin signaling are necessary and sufficient to specify lung progenitors in the foregut. Developmental cell *17*, 290-298.

Grassinger, J, Haylock, DN, Williams, B, Olsen, GH, Nilsson, SK (2010) Phenotypically identical hemopoietic stem cells isolated from different regions of BM have different biologic potential. Blood *116*, 3185-3196.

Gregory, MA, Phang, TL, Neviani, P, Alvarez-Calderon, F, Eide, CA, O'Hare, T, Zaberezhnyy, V, Williams, RT, Druker, BJ, Perrotti, D, Degregori, J (2010) Wnt/Ca2+/NFAT signaling maintains survival of Ph+ leukemia cells upon inhibition of Bcr-Abl. Cancer Cell *18*, 74-87.

Guo, J, Liu, M, Yang, D, Bouxsein, ML, Saito, H, Galvin, RJ, Kuhstoss, SA, Thomas, CC, Schipani, E, Baron, R, Bringhurst, FR, Kronenberg, HM (2010) Suppression of Wnt signaling by Dkk1 attenuates PTH-mediated stromal cell response and new bone formation. Cell Metab *11*, 161-171.

Guo, N, Hawkins, C, Nathans, J (2004) Frizzled6 controls hair patterning in mice. Proc Natl Acad Sci U S A *101*, 9277-9281.

Habas, R, Dawid, IB (2005) Dishevelled and Wnt signaling: is the nucleus the final frontier? J Biol *4*, 2.

Habas, R, Dawid, IB, He, X (2003) Coactivation of Rac and Rho by Wnt/Frizzled signaling is required for vertebrate gastrulation. Genes Dev *17*, 295-309.

Hardy, KM, Garriock, RJ, Yatskievych, TA, D'Agostino, SL, Antin, PB, Krieg, PA (2008) Non-canonical Wnt signaling through Wnt5a/b and a novel Wnt11 gene, Wnt11b, regulates cell migration during avian gastrulation. Developmental biology *320*, 391-401.

Haug, JS, He, XC, Grindley, JC, Wunderlich, JP, Gaudenz, K, Ross, JT, Paulson, A, Wagner, KP, Xie, Y, Zhu, R, Yin, T, Perry, JM, Hembree, MJ, Redenbaugh, EP, Radice, GL, Seidel, C, Li, L (2008) N-cadherin expression level distinguishes reserved versus primed states of hematopoietic stem cells. Cell stem cell *2*, 367-379.

Hauge, EM, Qvesel, D, Eriksen, EF, Mosekilde, L, Melsen, F (2001) Cancellous bone remodeling occurs in specialized compartments lined by cells expressing osteoblastic markers. J Bone Miner Res *16*, 1575-1582.

He, XC, Yin, T, Grindley, JC, Tian, Q, Sato, T, Tao, WA, Dirisina, R, Porter-Westpfahl, KS, Hembree, M, Johnson, T, Wiedemann, LM, Barrett, TA, Hood, L, Wu, H, Li, L (2007) PTEN-deficient intestinal stem cells initiate intestinal polyposis. Nature genetics *39*, 189-198.

He, XC, Zhang, J, Tong, WG, Tawfik, O, Ross, J, Scoville, DH, Tian, Q, Zeng, X, He, X, Wiedemann, LM, Mishina, Y, Li, L (2004a) BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. Nat Genet *36*, 1117-1121.

He, XC, Zhang, J, Tong, WG, Tawfik, O, Ross, J, Scoville, DH, Tian, Q, Zeng, X, He, X, Wiedemann, LM, Mishina, Y, Li, L (2004b) BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. Nature genetics *36*, 1117-1121.

Heinonen, KM, Vanegas, JR, Lew, D, Krosl, J, Perreault, C (2011) Wnt4 enhances murine hematopoietic progenitor cell expansion through a planar cell polarity-like pathway. PloS one *6*, e19279.

Horsley, V, Aliprantis, AO, Polak, L, Glimcher, LH, Fuchs, E (2008) NFATc1 balances quiescence and proliferation of skin stem cells. Cell *132*, 299-310.

Hsieh, JC, Kodjabachian, L, Rebbert, ML, Rattner, A, Smallwood, PM, Samos, CH, Nusse, R, Dawid, IB, Nathans, J (1999) A new secreted protein that binds to Wnt proteins and inhibits their activities. Nature *398*, 431-436.

Huang, T, Xie, Z, Wang, J, Li, M, Jing, N, Li, L (2011) Nuclear factor of activated T cells (NFAT) proteins repress canonical Wnt signaling via its interaction with Dishevelled (Dvl) protein and participate in regulating neural progenitor cell proliferation and differentiation. The Journal of biological chemistry *286*, 37399-37405.

Ikeya, M, Lee, SM, Johnson, JE, McMahon, AP, Takada, S (1997) Wnt signalling required for expansion of neural crest and CNS progenitors. Nature *389*, 966-970.

Iozzo, RV, Eichstetter, I, Danielson, KG (1995) Aberrant expression of the growth factor Wnt-5A in human malignancy. Cancer Res *55*, 3495-3499.

Ishikawa, T, Tamai, Y, Zorn, AM, Yoshida, H, Seldin, MF, Nishikawa, S, Taketo, MM (2001) Mouse Wnt receptor gene Fzd5 is essential for yolk sac and placental angiogenesis. Development (Cambridge, England) *128*, 25-33.

Ishitani, T, Kishida, S, Hyodo-Miura, J, Ueno, N, Yasuda, J, Waterman, M, Shibuya, H, Moon, RT, Ninomiya-Tsuji, J, Matsumoto, K (2003) The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca(2+) pathway to antagonize Wnt/beta-catenin signaling. Mol Cell Biol *23*, 131-139.

Janda, CY, Waghray, D, Levin, AM, Thomas, C, Garcia, KC (2012) Structural basis of Wnt recognition by Frizzled. Science (New York, NY *337*, 59-64.

Jeong, M, Piao, ZH, Kim, MS, Lee, SH, Yun, S, Sun, HN, Yoon, SR, Chung, JW, Kim, TD, Jeon, JH, Lee, J, Kim, HN, Choi, JY, Choi, I (2009) Thioredoxin-interacting protein regulates hematopoietic stem cell quiescence and mobilization under stress conditions. J Immunol *183*, 2495-2505.

Jho, E-h, Zhang, T, Domon, C, Joo, C-K, Freund, J-N, Costantini, F (2002) Wnt/{beta}-Catenin/Tcf Signaling Induces the Transcription of Axin2, a Negative Regulator of the Signaling Pathway. Mol Cell Biol *22*, 1172-1183.

Jordan, BK, Shen, JH, Olaso, R, Ingraham, HA, Vilain, E (2003) Wnt4 overexpression disrupts normal testicular vasculature and inhibits testosterone synthesis by repressing steroidogenic factor 1/beta-catenin synergy. Proc Natl Acad Sci U S A *100*, 10866-10871.

Kalajzic, I, Kalajzic, Z, Kaliterna, M, Gronowicz, G, Clark, SH, Lichtler, AC, Rowe, D (2002) Use of type I collagen green fluorescent protein transgenes to identify subpopulations of cells at different stages of the osteoblast lineage. J Bone Miner Res *17*, 15-25.

Kang, S, Bennett, CN, Gerin, I, Rapp, LA, Hankenson, KD, Macdougald, OA (2007) Wnt signaling stimulates osteoblastogenesis of mesenchymal precursors by suppressing CCAAT/enhancer-binding protein alpha and peroxisome proliferator-activated receptor gamma. J Biol Chem *282*, 14515-14524.

Kennerdell, JR, Carthew, RW (1998) Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. Cell *95*, 1017-1026.

Kiel, MJ, Acar, M, Radice, GL, Morrison, SJ (2008) Hematopoietic Stem Cells Do Not Depend on N-Cadherin to Regulate Their Maintenance. Cell stem cell.

Kiel, MJ, Yilmaz, OH, Iwashita, T, Terhorst, C, Morrison, SJ (2005) SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell *121*, 1109-1121.

Kimura, H, Usui, T, Tsubouchi, A, Uemura, T (2006) Potential dual molecular interaction of the Drosophila 7-pass transmembrane cadherin Flamingo in dendritic morphogenesis. Journal of cell science *119*, 1118-1129.

Kirstetter, P, Anderson, K, Porse, BT, Jacobsen, SE, Nerlov, C (2006) Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. Nat Immunol *7*, 1048-1056.

Kishida, S, Yamamoto, H, Hino, S, Ikeda, S, Kishida, M, Kikuchi, A (1999) DIX domains of Dvl and axin are necessary for protein interactions and their ability to regulate beta-catenin stability. Mol Cell Biol *19*, 4414-4422.

Koga, T, Matsui, Y, Asagiri, M, Kodama, T, de Crombrugghe, B, Nakashima, K, Takayanagi, H (2005) NFAT and Osterix cooperatively regulate bone formation. Nat Med *11*, 880-885.

Kohn, AD, Moon, RT (2005) Wnt and calcium signaling: beta-catenin-independent pathways. Cell Calcium *38*, 439-446.

Koschmieder, S, Gottgens, B, Zhang, P, Iwasaki-Arai, J, Akashi, K, Kutok, JL, Dayaram, T, Geary, K, Green, AR, Tenen, DG, Huettner, CS (2005) Inducible chronic phase of myeloid leukemia with expansion of hematopoietic stem cells in a transgenic model of BCR-ABL leukemogenesis. Blood *105*, 324-334.

Kuan, CY, Yang, DD, Samanta Roy, DR, Davis, RJ, Rakic, P, Flavell, RA (1999) The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. Neuron *22*, 667-676.

Kuo, CK, Tuan, RS (2008) Mechanoactive tenogenic differentiation of human MSCs. Tissue Eng Part A *14*, 1615-1627.

Kurayoshi, M, Oue, N, Yamamoto, H, Kishida, M, Inoue, A, Asahara, T, Yasui, W, Kikuchi, A (2006) Expression of Wnt-5a is correlated with aggressiveness of gastric cancer by stimulating cell migration and invasion. Cancer Res *66*, 10439-10448.

Lan, Y, Ryan, RC, Zhang, Z, Bullard, SA, Bush, JO, Maltby, KM, Lidral, AC, Jiang, R (2006) Expression of Wnt9b and activation of canonical Wnt signaling during midfacial morphogenesis in mice. Developmental dynamics : an official publication of the American Association of Anatomists *235*, 1448-1454.

Langmead, B, Trapnell, C, Pop, M, Salzberg, SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome biology *10*, R25.

Larsson, J, Karlsson, S (2005) The role of Smad signaling in hematopoiesis. Oncogene *24*, 5676-5692.

Leatherman, JL, Dinardo, S Germline self-renewal requires cyst stem cells and stat regulates niche adhesion in Drosophila testes. Nat Cell Biol *12*, 806-811.

Lee, JE, Wu, SF, Goering, LM, Dorsky, RI (2006) Canonical Wnt signaling through Lef1 is required for hypothalamic neurogenesis. Development (Cambridge, England) *133*, 4451-4461.

Lee, KH, Dinner, AR, Tu, C, Campi, G, Raychaudhuri, S, Varma, R, Sims, TN, Burack, WR, Wu, H, Wang, J, Kanagawa, O, Markiewicz, M, Allen, PM, Dustin, ML, Chakraborty, AK, Shaw, AS (2003) The immunological synapse balances T cell receptor signaling and degradation. Science (New York, NY *302*, 1218-1222.

Lejeune, S, Huguet, EL, Hamby, A, Poulsom, R, Harris, AL (1995) Wnt5a cloning, expression, and up-regulation in human primary breast cancers. Clin Cancer Res *1*, 215-222.

Levesque, JP, Helwani, FM, Winkler, IG (2010) The endosteal 'osteoblastic' niche and its role in hematopoietic stem cell homing and mobilization. Leukemia *24*, 1979-1992.

Li, C, Chen, H, Hu, L, Xing, Y, Sasaki, T, Villosis, MF, Li, J, Nishita, M, Minami, Y, Minoo, P (2008) Ror2 modulates the canonical Wnt signaling in lung epithelial cells through cooperation with Fzd2. BMC molecular biology *9*, 11.

Li, J, Sarosi, I, Cattley, RC, Pretorius, J, Asuncion, F, Grisanti, M, Morony, S, Adamu, S, Geng, Z, Qiu, W, Kostenuik, P, Lacey, DL, Simonet, WS, Bolon, B, Qian, X, Shalhoub, V, Ominsky, MS, Zhu Ke, H, Li, X, Richards, WG (2006) Dkk1-mediated inhibition of Wnt signaling in bone results in osteopenia. Bone *39*, 754-766.

Li, L, Clevers, H (2010) Coexistence of quiescent and active adult stem cells in mammals. Science (New York, NY *327*, 542-545.

Liang, H, Chen, Q, Coles, AH, Anderson, SJ, Pihan, G, Bradley, A, Gerstein, R, Jurecic, R, Jones, SN (2003) Wnt5a inhibits B cell proliferation and functions as a tumor suppressor in hematopoietic tissue. Cancer Cell *4*, 349-360.

Lindsley, RC, Gill, JG, Kyba, M, Murphy, TL, Murphy, KM (2006) Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. Development (Cambridge, England) *133*, 3787-3796.

Liu, G, Bafico, A, Aaronson, SA (2005) The mechanism of endogenous receptor activation functionally distinguishes prototype canonical and noncanonical Wnts. Mol Cell Biol *25*, 3475-3482.

Liu, P, Wakamiya, M, Shea, MJ, Albrecht, U, Behringer, RR, Bradley, A (1999) Requirement for Wnt3 in vertebrate axis formation. Nature genetics *22*, 361-365.

Logan, CY, Nusse, R (2004) The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol *20*, 781-810.

Longo, KA, Wright, WS, Kang, S, Gerin, I, Chiang, SH, Lucas, PC, Opp, MR, MacDougald, OA (2004) Wnt10b inhibits development of white and brown adipose tissues. The Journal of biological chemistry *279*, 35503-35509.

Louis, I, Heinonen, KM, Chagraoui, J, Vainio, S, Sauvageau, G, Perreault, C (2008) The signaling protein Wnt4 enhances thymopoiesis and expands multipotent hematopoietic progenitors through beta-catenin-independent signaling. Immunity *29*, 57-67.

Luis, TC, Naber, BA, Roozen, PP, Brugman, MH, de Haas, EF, Ghazvini, M, Fibbe, WE, van Dongen, JJ, Fodde, R, Staal, FJ (2011) Canonical wnt signaling regulates hematopoiesis in a dosage-dependent fashion. Cell stem cell *9*, 345-356.

Luo, J, Chen, J, Deng, ZL, Luo, X, Song, WX, Sharff, KA, Tang, N, Haydon, RC, Luu, HH, He, TC (2007) Wnt signaling and human diseases: what are the therapeutic implications? Lab Invest *87*, 97-103.

Lyden, D, Hattori, K, Dias, S, Costa, C, Blaikie, P, Butros, L, Chadburn, A, Heissig, B, Marks, W, Witte, L, Wu, Y, Hicklin, D, Zhu, Z, Hackett, NR, Crystal, RG, Moore, MA, Hajjar, KA, Manova, K, Benezra, R, Rafii, S (2001) Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med *7*, 1194-1201.

Lymperi, S, Ersek, A, Ferraro, F, Dazzi, F, Horwood, NJ (2011) Inhibition of osteoclast function reduces hematopoietic stem cell numbers in vivo. Blood *117*, 1540-1549.

Lymperi, S, Horwood, N, Marley, S, Gordon, MY, Cope, AP, Dazzi, F (2008) Strontium can increase some osteoblasts without increasing hematopoietic stem cells. Blood *111*, 1173-1181.

Lyuksyutova, AI, Lu, CC, Milanesio, N, King, LA, Guo, N, Wang, Y, Nathans, J, Tessier-Lavigne, M, Zou, Y (2003) Anterior-posterior guidance of commissural axons by Wnt-frizzled signaling. Science *302*, 1984-1988.

Ma, L, Wang, HY (2007) Mitogen-activated protein kinase p38 regulates the Wnt/cyclic GMP/Ca2+ non-canonical pathway. J Biol Chem *282*, 28980-28990.

Macian, F (2005) NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol *5*, 472-484.

Mahmood, A, Napoli, C, Aldahmash, A (2011) In vitro differentiation and maturation of human embryonic stem cell into multipotent cells. Stem cells international *2011*, 735420.

Malhotra, S, Kincade, PW (2009) Wnt-related molecules and signaling pathway equilibrium in hematopoiesis. Cell stem cell *4*, 27-36.

Martin, V, Agirre, X, Jimenez-Velasco, A, Jose-Eneriz, ES, Cordeu, L, Garate, L, Vilas-Zornoza, A, Castillejo, JA, Heiniger, A, Prosper, F, Torres, A, Roman-Gomez, J (2008) Methylation status of Wnt signaling pathway genes affects the clinical outcome of Philadelphia-positive acute lymphoblastic leukemia. Cancer Sci *99*, 1865-1868.

Matthews, HK, Marchant, L, Carmona-Fontaine, C, Kuriyama, S, Larrain, J, Holt, MR, Parsons, M, Mayor, R (2008) Directional migration of neural crest cells in vivo is regulated by Syndecan-4/Rac1 and non-canonical Wnt signaling/RhoA. Development *135*, 1771-1780.

Mendez-Ferrer, S, Michurina, TV, Ferraro, F, Mazloom, AR, Macarthur, BD, Lira, SA, Scadden, DT, Ma'ayan, A, Enikolopov, GN, Frenette, PS Mesenchymal and haematopoietic stem cells form a unique BM niche. Nature *466*, 829-834.

Mendez-Ferrer, S, Michurina, TV, Ferraro, F, Mazloom, AR, Macarthur, BD, Lira, SA, Scadden, DT, Ma'ayan, A, Enikolopov, GN, Frenette, PS (2010) Mesenchymal and haematopoietic stem cells form a unique BM niche. Nature *466*, 829-834.

Merkel, CE, Karner, CM, Carroll, TJ (2007) Molecular regulation of kidney development: is the answer blowing in the Wnt? Pediatr Nephrol *22*, 1825-1838.

Mignone, JL, Kukekov, V, Chiang, AS, Steindler, D, Enikolopov, G (2004) Neural stem and progenitor cells in nestin-GFP transgenic mice. The Journal of comparative neurology *469*, 311-324.

Miharada, K, Karlsson, G, Rehn, M, Rorby, E, Siva, K, Cammenga, J, Karlsson, S (2011) Cripto regulates hematopoietic stem cells as a hypoxic-niche-related factor through cell surface receptor GRP78. Cell stem cell *9*, 330-344.

Mikels, AJ, Nusse, R (2006) Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. PLoS Biol *4*, e115.

Mikkola, HK, Orkin, SH (2006) The journey of developing hematopoietic stem cells. Development (Cambridge, England) *133*, 3733-3744.

Miller, CL, Eaves, CJ (1997) Expansion in vitro of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability. Proceedings of the National Academy of Sciences of the United States of America *94*, 13648-13653.

Miller, SC, Jee, WS (1987) The bone lining cell: a distinct phenotype? Calcified tissue international *41*, 1-5.

Minta, A, Kao, JP, Tsien, RY (1989) Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. The Journal of biological chemistry *264*, 8171-8178.

Monticelli, S, Rao, A (2002) NFAT1 and NFAT2 are positive regulators of IL-4 gene transcription. European journal of immunology *32*, 2971-2978.

Moon, RT (1993) In pursuit of the functions of the Wnt family of developmental regulators: insights from Xenopus laevis. Bioessays *15*, 91-97.

Moon, RT, Kohn, AD, De Ferrari, GV, Kaykas, A (2004) WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet *5*, 691-701.

Morgan, R, El-Kadi, AM, Theokli, C (2003) Flamingo, a cadherin-type receptor involved in the Drosophila planar polarity pathway, can block signaling via the canonical wnt pathway in Xenopus laevis. The International journal of developmental biology *47*, 245-252.

Morrison, SJ, Wright, DE, Weissman, IL (1997) Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. Proceedings of the National Academy of Sciences of the United States of America *94*, 1908-1913.

Muller, MR, Sasaki, Y, Stevanovic, I, Lamperti, ED, Ghosh, S, Sharma, S, Gelinas, C, Rossi, DJ, Pipkin, ME, Rajewsky, K, Hogan, PG, Rao, A (2009) Requirement for balanced Ca/NFAT signaling in hematopoietic and embryonic development. Proceedings of the National Academy of Sciences of the United States of America *106*, 7034-7039.

Mulroy, T, McMahon, JA, Burakoff, SJ, McMahon, AP, Sen, J (2002) Wnt-1 and Wnt-4 regulate thymic cellularity. European journal of immunology *32*, 967-971.

Murdoch, B, Chadwick, K, Martin, M, Shojaei, F, Shah, KV, Gallacher, L, Moon, RT, Bhatia, M (2003) Wnt-5A augments repopulating capacity and primitive hematopoietic development of human blood stem cells in vivo. Proceedings of the National Academy of Sciences of the United States of America *100*, 3422-3427.

Nakano, T, Kodama, H, Honjo, T (1994) Generation of lymphohematopoietic cells from embryonic stem cells in culture. Science (New York, NY *265*, 1098-1101.

Naveiras, O, Nardi, V, Wenzel, PL, Hauschka, PV, Fahey, F, Daley, GQ (2009) Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. Nature *460*, 259-263.

Nelson, WJ, Nusse, R (2004) Convergence of Wnt, beta-catenin, and cadherin pathways. Science *303*, 1483-1487.

Nemeth, MJ, Mak, KK, Yang, Y, Bodine, DM (2009) beta-Catenin expression in the BM microenvironment is required for long-term maintenance of primitive hematopoietic cells. Stem cells (Dayton, Ohio) *27*, 1109-1119.

Nemeth, MJ, Topol, L, Anderson, SM, Yang, Y, Bodine, DM (2007) Wnt5a inhibits canonical Wnt signaling in hematopoietic stem cells and enhances repopulation. Proceedings of the National Academy of Sciences of the United States of America *104*, 15436-15441.

Nilsson, SK, Johnston, HM, Coverdale, JA (2001) Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. Blood *97*, 2293-2299.

Nusse, R, Varmus, HE (1982) Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell *31*, 99-109.

Nygren, MK, Dosen, G, Hystad, ME, Stubberud, H, Funderud, S, Rian, E (2007) Wnt3A activates canonical Wnt signalling in acute lymphoblastic leukaemia (ALL) cells and inhibits the proliferation of B-ALL cell lines. Br J Haematol *136*, 400-413.

Ohira, T, Gemmill, RM, Ferguson, K, Kusy, S, Roche, J, Brambilla, E, Zeng, C, Baron, A, Bemis, L, Erickson, P, Wilder, E, Rustgi, A, Kitajewski, J, Gabrielson, E, Bremnes, R, Franklin, W, Drabkin, HA (2003) WNT7a induces E-cadherin in lung cancer cells. Proceedings of the National Academy of Sciences of the United States of America *100*, 10429-10434.

Olson, DJ, Gibo, DM (1998) Antisense wnt-5a mimics wnt-1-mediated C57MG mammary epithelial cell transformation. Exp Cell Res *241*, 134-141.

Olson, DJ, Gibo, DM, Saggers, G, Debinski, W, Kumar, R (1997) Reversion of uroepithelial cell tumorigenesis by the ectopic expression of human wnt-5a. Cell Growth Differ *8*, 417-423.

Omatsu, Y, Sugiyama, T, Kohara, H, Kondoh, G, Fujii, N, Kohno, K, Nagasawa, T (2010) The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. Immunity *33*, 387-399.

Parkin, DM (2001) Global cancer statistics in the year 2000. Lancet Oncol *2*, 533-543.

Parr, BA, Cornish, VA, Cybulsky, MI, McMahon, AP (2001) Wnt7b regulates placental development in mice. Developmental biology *237*, 324-332.

Perry, JM, He, XC, Sugimura, R, Grindley, JC, Haug, JS, Ding, S, Li, L (2011a) Cooperation between both Wnt/{beta}-catenin and PTEN/PI3K/Akt signaling promotes primitive hematopoietic stem cell self-renewal and expansion. Genes & development *25*, 1928-1942.

Perry, JM, He, XC, Sugimura, R, Grindley, JC, Haug, JS, Ding, S, Li, L (2011b) Cooperation between both Wnt/{beta}-catenin and PTEN/PI3K/Akt signaling promotes primitive hematopoietic stem cell self-renewal and expansion. Genes Dev.

Perry, JM, Li, L (2007) Disrupting the stem cell niche: good seeds in bad soil. Cell *129*, 1045-1047.

Perry, JM, Li, L (2012) To be or not to be a stem cell: dissection of cellular and molecular components of haematopoietic stem cell niches. The EMBO journal *31*, 1060-1061.

Pinto, D, Gregorieff, A, Begthel, H, Clevers, H (2003) Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. Genes & development *17*, 1709-1713.

Piters, E, Boudin, E, Van Hul, W (2008) Wnt signaling: a win for bone. Arch Biochem Biophys *473*, 112-116.

Pittenger, MF, Mackay, AM, Beck, SC, Jaiswal, RK, Douglas, R, Mosca, JD, Moorman, MA, Simonetti, DW, Craig, S, Marshak, DR (1999) Multilineage potential of adult human MSCs. Science (New York, NY *284*, 143-147.

Planutis, K, Planutiene, M, Moyer, MP, Nguyen, AV, Perez, CA, Holcombe, RF (2007) Regulation of norrin receptor frizzled-4 by Wnt2 in colon-derived cells. BMC cell biology *8*, 12.

Pukrop, T, Klemm, F, Hagemann, T, Gradl, D, Schulz, M, Siemes, S, Trumper, L, Binder, C (2006) Wnt 5a signaling is critical for macrophage-induced invasion of breast cancer cell lines. Proc Natl Acad Sci U S A *103*, 5454-5459.

Raaijmakers, MH, Mukherjee, S, Guo, S, Zhang, S, Kobayashi, T, Schoonmaker, JA, Ebert, BL, Al-Shahrour, F, Hasserjian, RP, Scadden, EO, Aung, Z, Matza, M, Merkenschlager, M, Lin, C, Rommens, JM, Scadden, DT (2010) Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. Nature *464*, 852-857.

Raggatt, LJ, Partridge, NC (2010) Cellular and molecular mechanisms of bone remodeling. The Journal of biological chemistry *285*, 25103-25108.

Ranheim, EA, Kwan, HC, Reya, T, Wang, YK, Weissman, IL, Francke, U (2005) Frizzled 9 knock-out mice have abnormal B-cell development. Blood *105*, 2487-2494.

Rasmussen, JT, Deardorff, MA, Tan, C, Rao, MS, Klein, PS, Vetter, ML (2001) Regulation of eye development by frizzled signaling in Xenopus. Proceedings of the National Academy of Sciences of the United States of America *98*, 3861-3866.

Renstrom, J, Istvanffy, R, Gauthier, K, Shimono, A, Mages, J, Jardon-Alvarez, A, Kroger, M, Schiemann, M, Busch, DH, Esposito, I, Lang, R, Peschel, C, Oostendorp, RA (2009) Secreted frizzled-related protein 1 extrinsically regulates cycling activity and maintenance of hematopoietic stem cells. Cell stem cell *5*, 157-167.

Reya, T, Clevers, H (2005) Wnt signalling in stem cells and cancer. Nature *434*, 843-850.

Reya, T, Duncan, AW, Ailles, L, Domen, J, Scherer, DC, Willert, K, Hintz, L, Nusse, R, Weissman, IL (2003) A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature *423*, 409-414.

Reya, T, Morrison, SJ, Clarke, MF, Weissman, IL (2001) Stem cells, cancer, and cancer stem cells. Nature *414*, 105-111.

Roman-Gomez, J, Jimenez-Velasco, A, Cordeu, L, Vilas-Zornoza, A, San Jose-Eneriz, E, Garate, L, Castillejo, JA, Martin, V, Prosper, F, Heiniger, A, Torres, A, Agirre, X (2007) WNT5A, a putative tumour suppressor of lymphoid malignancies, is inactivated by aberrant methylation in acute lymphoblastic leukaemia. Eur J Cancer *43*, 2736-2746.

Rousselot, P, Huguet, F, Rea, D, Legros, L, Cayuela, JM, Maarek, O, Blanchet, O, Marit, G, Gluckman, E, Reiffers, J, Gardembas, M, Mahon, FX (2007) Imatinib

mesylate discontinuation in patients with chronic myelogenous leukemia in complete molecular remission for more than 2 years. Blood *109*, 58-60.

Sancho, E, Batlle, E, Clevers, H (2004) Signaling pathways in intestinal development and cancer. Annual review of cell and developmental biology *20*, 695-723.

Saneyoshi, T, Kume, S, Amasaki, Y, Mikoshiba, K (2002) The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in Xenopus embryos. Nature *417*, 295-299.

Schaniel, C, Sirabella, D, Qiu, J, Niu, X, Lemischka, IR, Moore, KA (2011) Wnt-inhibitory factor 1 dysregulation of the BM niche exhausts hematopoietic stem cells. Blood.

Scheller, M, Huelsken, J, Rosenbauer, F, Taketo, MM, Birchmeier, W, Tenen, DG, Leutz, A (2006) Hematopoietic stem cell and multilineage defects generated by constitutive [beta]-catenin activation. Nature immunology *7*, 1037-1047.

Schlessinger, K, Hall, A, Tolwinski, N (2009) Wnt signaling pathways meet Rho GTPases. Genes Dev *23*, 265-277.

Schlessinger, K, McManus, EJ, Hall, A (2007) Cdc42 and noncanonical Wnt signal transduction pathways cooperate to promote cell polarity. J Cell Biol *178*, 355-361.

Schmidt, C, McGonnell, IM, Allen, S, Otto, A, Patel, K (2007) Wnt6 controls amniote neural crest induction through the non-canonical signaling pathway. Developmental dynamics : an official publication of the American Association of Anatomists *236*, 2502-2511.

Schofield, R (1978) The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells *4*, 7-25.

Scoville, DH, Sato, T, He, XC, Li, L (2008) Current view: intestinal stem cells and signaling. Gastroenterology *134*, 849-864.

Semenov, MV, Habas, R, Macdonald, BT, He, X (2007) SnapShot: Noncanonical Wnt Signaling Pathways. Cell *131*, 1378.

Sen, B, Styner, M, Xie, Z, Case, N, Rubin, CT, Rubin, J (2009) Mechanical loading regulates NFATc1 and beta-catenin signaling through a GSK3beta control node. J Biol Chem *284*, 34607-34617.

Sharma, RP, Chopra, VL (1976) Effect of the Wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster. Developmental biology *48*, 461-465.

Sheldahl, LC, Slusarski, DC, Pandur, P, Miller, JR, Kuhl, M, Moon, RT (2003) Dishevelled activates Ca2+ flux, PKC, and CamKII in vertebrate embryos. J Cell Biol *161*, 769-777.

Shima, Y, Kawaguchi, SY, Kosaka, K, Nakayama, M, Hoshino, M, Nabeshima, Y, Hirano, T, Uemura, T (2007) Opposing roles in neurite growth control by two seven-pass transmembrane cadherins. Nat Neurosci *10*, 963-969.

Shima, Y, Kengaku, M, Hirano, T, Takeichi, M, Uemura, T (2004) Regulation of dendritic maintenance and growth by a mammalian 7-pass transmembrane cadherin. Developmental cell *7*, 205-216.

Slusarski, DC, Yang-Snyder, J, Busa, WB, Moon, RT (1997) Modulation of embryonic intracellular Ca2+ signaling by Wnt-5A. Dev Biol *182*, 114-120.

Sousa, KM, Villaescusa, JC, Cajanek, L, Ondr, JK, Castelo-Branco, G, Hofstra, W, Bryja, V, Palmberg, C, Bergman, T, Wainwright, B, Lang, RA, Arenas, E (2010) Wnt2 regulates progenitor proliferation in the developing ventral midbrain. The Journal of biological chemistry *285*, 7246-7253.

Spater, D, Hill, TP, Gruber, M, Hartmann, C (2006) Role of canonical Wnt-signalling in joint formation. European cells & materials *12*, 71-80.

Sugimura, R, Li, L (2010) Shifting in balance between osteogenesis and adipogenesis substantially influences hematopoiesis. J Mol Cell Biol *2*, 61-62.

Sugiyama, T, Kohara, H, Noda, M, Nagasawa, T (2006) Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in BM stromal cell niches. Immunity *25*, 977-988.

Takada, I, Mihara, M, Suzawa, M, Ohtake, F, Kobayashi, S, Igarashi, M, Youn, MY, Takeyama, K, Nakamura, T, Mezaki, Y, Takezawa, S, Yogiashi, Y, Kitagawa, H, Yamada, G, Takada, S, Minami, Y, Shibuya, H, Matsumoto, K, Kato, S (2007) A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. Nature cell biology *9*, 1273-1285.

Tissir, F, Bar, I, Jossin, Y, De Backer, O, Goffinet, AM (2005) Protocadherin Celsr3 is crucial in axonal tract development. Nat Neurosci *8*, 451-457.

Tissir, F, De-Backer, O, Goffinet, AM, Lambert de Rouvroit, C (2002) Developmental expression profiles of Celsr (Flamingo) genes in the mouse. Mech Dev *112*, 157-160.

Tissir, F, Qu, Y, Montcouquiol, M, Zhou, L, Komatsu, K, Shi, D, Fujimori, T, Labeau, J, Tyteca, D, Courtoy, P, Poumay, Y, Uemura, T, Goffinet, AM Lack of cadherins Celsr2 and Celsr3 impairs ependymal ciliogenesis, leading to fatal hydrocephalus. Nat Neurosci *13*, 700-707.

Tissir, F, Qu, Y, Montcouquiol, M, Zhou, L, Komatsu, K, Shi, D, Fujimori, T, Labeau, J, Tyteca, D, Courtoy, P, Poumay, Y, Uemura, T, Goffinet, AM (2010) Lack of cadherins Celsr2 and Celsr3 impairs ependymal ciliogenesis, leading to fatal hydrocephalus. Nat Neurosci *13*, 700-707.

Trapnell, C, Pachter, L, Salzberg, SL (2009) TopHat: discovering splice junctions with RNA-Seq. Bioinformatics *25*, 1105-1111.

Trapnell, C, Williams, BA, Pertea, G, Mortazavi, A, Kwan, G, van Baren, MJ, Salzberg, SL, Wold, BJ, Pachter, L (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nature biotechnology *28*, 511-515.

Tumbar, T, Guasch, G, Greco, V, Blanpain, C, Lowry, WE, Rendl, M, Fuchs, E (2004) Defining the epithelial stem cell niche in skin. Science (New York, NY *303*, 359-363.

Uccelli, A, Moretta, L, Pistoia, V (2008) MSCs in health and disease. Nat Rev Immunol.

Usui, T, Shima, Y, Shimada, Y, Hirano, S, Burgess, RW, Schwarz, TL, Takeichi, M, Uemura, T (1999) Flamingo, a seven-pass transmembrane cadherin, regulates planar cell polarity under the control of Frizzled. Cell *98*, 585-595.

van Amerongen, R, Berns, A (2006) Knockout mouse models to study Wnt signal transduction. Trends Genet *22*, 678-689.

van de Wetering, M, Sancho, E, Verweij, C, de Lau, W, Oving, I, Hurlstone, A, van der Horn, K, Batlle, E, Coudreuse, D, Haramis, AP, Tjon-Pon-Fong, M, Moerer, P, van den Born, M, Soete, G, Pals, S, Eilers, M, Medema, R, Clevers, H (2002) The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell *111*, 241-250.

van Es, JH, Jay, P, Gregorieff, A, van Gijn, ME, Jonkheer, S, Hatzis, P, Thiele, A, van den Born, M, Begthel, H, Brabletz, T, Taketo, MM, Clevers, H (2005) Wnt signalling induces maturation of Paneth cells in intestinal crypts. Nature cell biology *7*, 381-386.

van Gijn, ME, Daemen, MJ, Smits, JF, Blankesteijn, WM (2002) The wnt-frizzled cascade in cardiovascular disease. Cardiovasc Res *55*, 16-24.

Veeman, MT, Axelrod, JD, Moon, RT (2003) A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. Developmental cell *5*, 367-377.

Vijayaragavan, K, Szabo, E, Bosse, M, Ramos-Mejia, V, Moon, RT, Bhatia, M (2009) Noncanonical Wnt signaling orchestrates early developmental events toward hematopoietic cell fate from human embryonic stem cells. Cell Stem Cell *4*, 248-262.

Visnjic, D, Kalajzic, Z, Rowe, DW, Katavic, V, Lorenzo, J, Aguila, HL (2004) Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. Blood *103*, 3258-3264.

Wallingford, JB, Habas, R (2005) The developmental biology of Dishevelled: an enigmatic protein governing cell fate and cell polarity. Development (Cambridge, England) *132*, 4421-4436.

Wallingford, JB, Vogeli, KM, Harland, RM (2001) Regulation of convergent extension in Xenopus by Wnt5a and Frizzled-8 is independent of the canonical Wnt pathway. The International journal of developmental biology *45*, 225-227.

Wang, Y, Guo, N, Nathans, J (2006) The role of Frizzled3 and Frizzled6 in neural tube closure and in the planar polarity of inner-ear sensory hair cells. J Neurosci *26*, 2147-2156.

Wang, Y, Thekdi, N, Smallwood, PM, Macke, JP, Nathans, J (2002) Frizzled-3 is required for the development of major fiber tracts in the rostral CNS. J Neurosci *22*, 8563-8573.

Wang, YJ, Chen, GH, Hu, XY, Lu, YP, Zhou, JN, Liu, RY (2005a) The expression of calcium/calmodulin-dependent protein kinase II-alpha in the hippocampus of patients with Alzheimer's disease and its links with AD-related pathology. Brain Res *1031*, 101-108.

Wang, Z, Shu, W, Lu, MM, Morrisey, EE (2005b) Wnt7b activates canonical signaling in epithelial and vascular smooth muscle cells through interactions with Fzd1, Fzd10, and LRP5. Molecular and cellular biology *25*, 5022-5030.

Weeraratna, AT, Jiang, Y, Hostetter, G, Rosenblatt, K, Duray, P, Bittner, M, Trent, JM (2002) Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. Cancer Cell *1*, 279-288.

Weissman, IL, Anderson, DJ, Gage, F (2001) Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. Annual review of cell and developmental biology *17*, 387-403.

Weston, CR, Wong, A, Hall, JP, Goad, ME, Flavell, RA, Davis, RJ (2003) JNK initiates a cytokine cascade that causes Pax2 expression and closure of the optic fissure. Genes Dev *17*, 1271-1280.

Wilson, A, Laurenti, E, Oser, G, van der Wath, RC, Blanco-Bose, W, Jaworski, M, Offner, S, Dunant, CF, Eshkind, L, Bockamp, E, Lio, P, Macdonald, HR, Trumpp, A (2008) Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. Cell *135*, 1118-1129.

Wilson, A, Trumpp, A (2006) Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol *6*, 93-106.

Xie, T, Spradling, AC (1998) Decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary. Cell *94*, 251-260.

Xie, Y, Yin, T, Wiegraebe, W, He, XC, Miller, D, Stark, D, Perko, K, Alexander, R, Schwartz, J, Grindley, JC, Park, J, Haug, JS, Wunderlich, JP, Li, H, Zhang, S, Johnson, T, Feldman, RA, Li, L (2009) Detection of functional haematopoietic stem cell niche using real-time imaging. Nature *457*, 97-101.

Yamanaka, H, Moriguchi, T, Masuyama, N, Kusakabe, M, Hanafusa, H, Takada, R, Takada, S, Nishida, E (2002) JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. EMBO Rep *3*, 69-75.

Yamazaki, S, Ema, H, Karlsson, G, Yamaguchi, T, Miyoshi, H, Shioda, S, Taketo, MM, Karlsson, S, Iwama, A, Nakauchi, H (2011) Nonmyelinating schwann cells maintain hematopoietic stem cell hibernation in the BM niche. Cell *147*, 1146-1158.

Yang, FC, Atkinson, SJ, Gu, Y, Borneo, JB, Roberts, AW, Zheng, Y, Pennington, J, Williams, DA (2001) Rac and Cdc42 GTPases control hematopoietic stem cell shape, adhesion, migration, and mobilization. Proceedings of the National Academy of Sciences of the United States of America *98*, 5614-5618.

Yang, L, Bryder, D, Adolfsson, J, Nygren, J, Mansson, R, Sigvardsson, M, Jacobsen, SE (2005) Identification of Lin(-)Sca1(+)kit(+)CD34(+)Flt3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. Blood *105*, 2717-2723.

Yang, L, Wang, L, Geiger, H, Cancelas, JA, Mo, J, Zheng, Y (2007) Rho GTPase Cdc42 coordinates hematopoietic stem cell quiescence and niche interaction in the BM. Proceedings of the National Academy of Sciences of the United States of America *104*, 5091-5096.

Ye, L, Fan, Z, Yu, B, Chang, J, Al Hezaimi, K, Zhou, X, Park, NH, Wang, CY (2012) Histone Demethylases KDM4B and KDM6B Promotes Osteogenic Differentiation of Human MSCs. Cell stem cell *11*, 50-61.

Ye, X, Wang, Y, Rattner, A, Nathans, J (2011) Genetic mosaic analysis reveals a major role for frizzled 4 and frizzled 8 in controlling ureteric growth in the developing kidney. Development (Cambridge, England) *138*, 1161-1172.

Yilmaz, OH, Valdez, R, Theisen, BK, Guo, W, Ferguson, DO, Wu, H, Morrison, SJ (2006a) Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. Nature *441*, 475-482.

Yilmaz, OH, Valdez, R, Theisen, BK, Guo, W, Ferguson, DO, Wu, H, Morrison, SJ (2006b) Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. Nature *441*, 475-482.

Ying, Q-L, Wray, J, Nichols, J, Batlle-Morera, L, Doble, B, Woodgett, J, Cohen, P, Smith, A (2008) The ground state of embryonic stem cell self-renewal. Nature *453*, 519-523.

Yoshihara, H, Arai, F, Hosokawa, K, Hagiwara, T, Takubo, K, Nakamura, Y, Gomei, Y, Iwasaki, H, Matsuoka, S, Miyamoto, K, Miyazaki, H, Takahashi, T, Suda, T (2007) Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. Cell stem cell *1*, 685-697.

You, L, He, B, Uematsu, K, Xu, Z, Mazieres, J, Lee, A, McCormick, F, Jablons, DM (2004) Inhibition of Wnt-1 signaling induces apoptosis in beta-catenin-deficient mesothelioma cells. Cancer research *64*, 3474-3478.

Zhang, J, Grindley, JC, Yin, T, Jayasinghe, S, He, XC, Ross, JT, Haug, JS, Rupp, D, Porter-Westpfahl, KS, Wiedemann, LM, Wu, H, Li, L (2006) PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. Nature *441*, 518-522.

Zhang, J, Niu, C, Ye, L, Huang, H, He, X, Tong, WG, Ross, J, Haug, J, Johnson, T, Feng, JQ, Harris, S, Wiedemann, LM, Mishina, Y, Li, L (2003a) Identification of the haematopoietic stem cell niche and control of the niche size. Nature *425*, 836-841.

Zhang, T, Maier, LS, Dalton, ND, Miyamoto, S, Ross, J, Jr., Bers, DM, Brown, JH (2003b) The deltaC isoform of CaMKII is activated in cardiac hypertrophy and induces dilated cardiomyopathy and heart failure. Circ Res *92*, 912-919.

Zou, P, Yoshihara, H, Hosokawa, K, Tai, I, Shinmyozu, K, Tsukahara, F, Maru, Y, Nakayama, K, Nakayama, KI, Suda, T (2011) p57(Kip2) and p27(Kip1) Cooperate to Maintain Hematopoietic Stem Cell Quiescence through Interactions with Hsc70. Cell stem cell *9*, 247-261.

APPENDIX

Primers	Sequence
Flamingo (Celsr2) Fwd	TGTACAGCAGTAGCCCGTTACG
Flamingo (Celsr2) Rev	CGCCAGCGCTTCCACTT
Frizzled1 Fwd	CAAGGTTTACGGGCTCATGT
Frizzled1 Rev	GTAACAGCCGGACAGGAAAA
Frizzled2 Fwd	GAGGTGCATCAGTTCTACCC
Frizzled2 Rev	ATGGCCTGCTCCAGCACT
Frizzled3 Fwd	GCAGATAGGTGGGCACAGTT
Frizzled3 Rev	AAAGAAATGGCCGAAAATCC
Frizzled4 Fwd	CACGCCGCTCATCCAGTA
Frizzled4 Rev	GCCGATGGGGATGTTGAT
Frizzled5 Fwd	CAACCATGACACGCAGGAC
Frizzled5 Rev	GGGCGTGTACATAGAGCACA
Frizzled6 Fwd	TTCCCTAACCTGATGGGTCA
Frizzled6 Rev	ACATTTCAATGTTTGGTGAACA
Frizzled7 Fwd	ATATCGCCTACAACCAGACCATCC
Frizzled7 Rev	AAGGAACGGCACGGAGGAATG
Frizzled8 Fwd	ATGGAGTGGGGTTACCTGTTG
Frizzled8 Rev	CACCGTGATCTCTTGGCAC
Frizzled9 Fwd	GTCCGCGTTGTGTTTCTTCT
Frizzled9 Rev	CAGACCCTCCTGGATCACAT
Frizzled10 Fwd	GTACCCCGAACGTCCTATCA
Frizzled10 Rev	GTGCTCTCCAGTCCTTCCTG
Cox2 Fwd	CCACCACTACTGCCACCTC
Cox2 Rev	TGGTCAAATCCTGTGCTCAT
IL2 Fwd	AACCTGAAACTCCCCAGGAT
IL2 Rev	CGCAGAGGTCCAAGTTCATC
CD34 Fwd	ACAGGAGAATGCAGGTCCAC
CD34 Rev	TGGTAGGAACTGATGGGGATA
Ifnγ Fwd	GCTTTGCAGCTCTTCCTCAT
Ifnγ Rev	TTTTGCCAGTTCCTCCAGAT
p57 Fwd	ACAGGACAAGCGATCCAGAC
p57 Rev	GCGCTATCACTGGGAAGGT
Pten Fwd	AGATCGTTAGCAGAAACAAAAGG
Pten Rev	TCTGCAGGAAATCCCATAGC
Txnip Fwd	GCAGTGCAAACAGACTTTGG
Txnip Rev	AGCTCGAAGCCGAACTTGTA
Tnfα Fwd	CCACCACGCTCTTCTGTCTA
Tnfα Rev	AGGGTCTGGGCCATAGAACT
N-cadherin Fwd	AGCGCAGTCTTACCGAAGG
N-cadherin Rev	TCGCTGCTTTCATACTGAACTTT
Osterix Fwd	ATGGCGTCCTCTCTGCTTG
Osterix Rev	TGAAAGGTCAGCGTATGGCTT
Runx2 Fwd	CGGCCCTCCCTGAACTCT
Runx2 Rev	TGCCTGCCTGGGATCTGTA

Rpl19 Fwd	ATGAGTATGCTCAGGCTACAGA
Rpl19 Rev	GCATTGGCGATTTCATTGGTC
Gapdh Fwd	TGGCAAAGTGGAGATTGTTGCC
Gapdh Rev	AAGATGGTGATGGGCTTCCCG

TaqMan assays	Serial number (Applied Biosystems)
Wnt1	Mm-01300555_g1
Wnt2	Mm-00470018_m1
Wnt2b	Mm-00437330_m1
Wnt3	Mm-00437336_m1
Wnt3a	Mm-00437337_m1
Wnt4	Mm-01194003_m1
Wnt5a	Mm-00437347_m1
Wnt5b	Mm-01183986_m1
Wnt6	Mm-00437353_m1
Wnt7a	Mm-00437354_m1
Wnt7b	Mm-01301717_m1
Wnt8a	Mm-00436822_m1
Wnt8b	Mm-00442107_m1
Wnt9a	Mm-00460518_m1
Wnt9b	Mm-00457102_m1
Wnt10a	Mm-00437325_m1
Wnt10b	Mm-00442104_m1
Wnt11	Mm-00437328_m1
Wnt16	Mm-00446420_m1
Dkk1	Mm-00438422_m1
Dkk3	Mm-00443800_m1
Sfrp4	Mm-00840104_m1
Wif1	Mm-00442355_m1
Axin2	Mm-00443610_m1
N-cadherin	Mm-00483213_m1